Studying the role of β1,3-*N*-acetylglucosaminyltransferases in the mouse

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Table of Contents

Abbreviations*:

Asn	Asparagine
B3gnt1-5	β1,3-*N*-acetylglucosaminyltransferase
CAZy	**C**arbohydrate-**A**ctive en**zy**mes (classification system for GTs)
Cer	Ceramide
Dol-P	Dolichol-phosphate
ER	Endoplasmic reticulum
Fuc	Fucose
GAG	Glycosaminoglycan
Gal	Galactose
Gal-Cer	Galactosylceramide
GalNAc	*N*-acetylgalactosamine
Glc	Glucose
GlcA	Glucuronic acid
Glc-Cer	Glucosylceramide
GlcNAc	*N*-acetylglucosamine
Gn-RH neurons	Gonadotropin-releasing hormone
GPI	Glycophosphoinositol
GT	Glycosyltransferase
HBGA	histo-blood group antigens
Lac-Cer	Lactosylceramide
Le^x	Lewis x antigen
LOS	Lipooligosaccharide
Man	Mannose
NV	Norwalk virus
OB	Olfactory bulb
OE	Olfactory epithelium
PCR	Polymerase chain reaction
PTM	Post translational modification
RBC	red blood cell
Ser	Serine
Sia	Sialic acid
SGGL	Sulfoglucuronylglycolipids
SSEA	Stage specific embryonic antigens
Thr	Threonine
UDP	Uridine diphosphate
VNO	Vomeronasal organ
Xyl	Xylose

* All abbreviations used are found in their complete forms the first time they are used in the text. For further explanations or definitions of abbreviations please see relevant text.

1. Thesis summary

Glycosyltransferases (GTs) are enzymes which catalyze the synthesis of glycans on various biological substrates. As modifiers of lipids and proteins, in or outside of the cell, glycans direct and coordinate intra- and intercellular communication. In addition glycans are often the first molecules within an organism to come into contact with invading pathogens and act as docking or entry ports. Whether as structural modifiers or signaling molecules, glycans play an essential role in a wide variety of biological processes. So far much of what is known GTs stems from *in vitro* biochemical assays or cell culture experiments. In an effort to gain a better understanding of these enzymes and the processes they influence *in vivo* data are necessary. The availability of murine embryonic stem cells makes the genetic manipulation of mice possible, and allows the targeted mutants to serve as mammalian model organisms in order to study specific genes.

In this study two genes belonging to the β1,3-*N*-acetylglucosaminyltransferase (*B3gnt*) family were disrupted in the mouse. The *B3gnt* gene family encodes GTs which catalyze the addition of *N*-acetylglucosamine (GlcNAc) to terminal galactose (Gal) or *N*-acetylgalactosamine (GalNAc) on specific substrates in the β1,3 bond configuration. To this end, two mouse strains with targeted deletions for the *B3gnt1* and *B3gnt5* were created and examined. Previous investigations in the wildtype mouse showed the expression of *B3gnt1* is ubiquitous while *B3gnt5* reveals a more restricted expression pattern. *In vitro* enzyme activity assays of β3GNT1 and β3GNT5 showed they had very different acceptor substrate specificities. β3GNT1 has been described as a enzyme able to modify terminal Gal on N-glycans, O-glycans, glycolipids, and glycosaminoglycan chains in all tissues. The activity of β3GNT5 was limited to the elongation of lacto-series glycolipids in a set of select tissues such as the placenta, testes, and spleen. β3GNT5 expression was also documented in the developing embryo. The following study describes two mice missing GTs with similar catalytic activity with markedly different phenotypic effects.

Generally, we expected a more pronounced phenotype detectable in the *B3gnt1* null mice than in the *B3gnt5* mice due to its ubiquitous expression. For this portion of the study an existing *B3gnt1* null mouse line was used to examine phenotypic effects caused by the lack of *B3gnt1*. Contrary to what we expected, the disruption of *B3gnt1* showed no overt physical phenotype. Upon closer examination with collaborators we were able to show

that there was some impaired olfactory development. Breeding the null animals was almost impossible. Investigations into the fertility of these null animals followed. The low fertility rate of male mice was finally linked to impaired male sexual interaction with ovulating females and the data collected used to create the first manuscript found in this dissertation.

The second targeted mutant mouse was completed during the course of this project. A targeting vector was constructed and electroporated into murine embryonic stem (ES) cells. These clones in turn were used to reproduce two independent chimeric males which both exhibited germ line transmission of the null allele. Examination of the role of the β3GNT5 was cut short due to its effects on early embryonic development. Unfortunately, the disruption of this glycolipid modifying enzyme resulted in early embryonic death of the mice. We were not able to detect mice with homozygous null mutation as early as the blastocyst stage. The second manuscript describes the results obtained from this second study.

Zusammenfassung

Die katalytische Funktion der Glykosyltransferasen (GT) ermöglicht die Modifikation von verschiedenen biologischen Substraten mit Glykanen. Angehängt an Proteine und Lipide können Glykane komplexe Vorgänge innerhalb sowie ausserhalb der Zelle regulieren und beeinflussen. Glykane sind auch oft die ersten Moleküle, welche mit Mikroorganismen in Kontakt kommen, und werden deshalb als Andock- oder als Eingangstellen benutzt. Aufgrund dieser diversen Rollen, die von strukturellen Modifikationen bis hin zur Signaltransduktion reichen, nehmen die Glykane eine essentielle Rolle in biologischen Abläufen ein. Um die GT und die Vorgänge, die von diesen Enzymen abhängig sind, *in vivo* besser zu verstehen und zu beschreiben, bietet sich die Maus als ideales Säugetier-Modell an. Seit embryonale Stammzellen von Mäusen verfügbar sind, kann man diese nutzen um spezifische Gene gezielt zu mutieren und zu untersuchen.

In dieser Studie wurden zwei Mitglieder der β1,3-*N*-acteylglukosaminyltransferasen (*B3gnt*) Gen-Familie in der Maus untersucht. Die *B3gnt* Gen-Familie codiert mehrere GT welche die Modifikation einer terminalen Galaktose (Gal) oder *N*-acetylgalalaktose (GalNAc) mit einem *N*-acetylglukosamin (GlcNAc) katalysiert und in der β1,3 Konfiguration verbindet. Mauslinien wurden hergestellt, in welchen die Gene *B3gnt1* und *B3gnt5* gezielt zerstört wurden. Die zwei resultierenden Linien wurden dann auf auffällige Phänotypen untersucht. Frühere Studien zeigten, dass *B3gnt1* ubiquitär in Organen von erwachsenen Mäusen exprimiert wird. Weitere *in vitro* Analysen zeigten, dass das β3GNT1 Enzym alle N-und O-Glykane, Glycolipide, sowie Glycosaminoglykane als Substrat verwenden kann. Das Expressionsmuster des *B3gnt5* Genes ist restriktiver, da dessen Aktivität sich auf Glykolipide der Lacto-Serie beschränkt. Und diese Expression ist nur in ausgewählten Organen, wie die Hoden, Plazenta, und die Milz nachzuweisen. Ausserdem konnte man *B3gnt5* in mehreren Entwicklungsstadien von Mausembryos nachweisen. Die Untersuchungen in dieser Arbeit beschreiben die zwei Maus Linien und die unterschiedlichen Folgen, welche die Ausschaltung der beiden Gene verursachen, auch wenn die Donor- und Substratspezifitäten der beiden Gene ähnlich sind.

Generell erwartet man beim Ausschalten von Genen, die ubiquitär exprimiert sind, einen ausgeprägten Phänotyp. Im ersten Teil dieser Arbeit wurde eine bereits existierende Mauslinie ohne aktives *B3gnt1* Gen untersucht. Im Gegensatz zu den aufgrund des

Expressions- und Aktivitätsmuster des Gens erwarteten Auswirkungen fiel der Phänotyp vergleichsweise mild aus. Durch gezielte Untersuchungen kamen wir zum Schluss, dass die Tiere eine Missbildung des olfaktorischen Organs aufweisen, welche während der frühen Entwicklung auftritt. Das Züchten der homozygot-null Tiere erwies sich als äusserst schwierig. Die niedrige Fruchtbarkeit der männlichen Mäuse wurde als mögliche Langzeitfolge der olfaktorische Störung interpretiert. Im ersten Manuskript wird beschrieben, wie gezeigt wurde, dass die Männchen keine sexuellen Interaktionen mit ovulierenden Weibchen anstreben. Dies wurde auf ein fehlendes Erkennen der Weibchen in den Verhaltenstests zurückgeführt.

Die zweite Mauslinie wurde als Teil dieser Arbeit hergestellt. Mit Hilfe eines Vektors, welcher gezielt mit dem *B3gnt5* Gen rekombinieren kann, wurden mehrere heterozygote embryonale Stammzelllinien kreiert. Zur Herstellung eines transgenen Tieres konnten diese Klone in einem weiteren Schritt in Mausblastozysten eingepflanzt werden. Zwei unabhängige heterozygote Männchen, welche das zerstörte *B3gnt5* Allel weitervererben konnten, resultierten aus erfolgreich rekombinierten Blastozysten. Leider konnten keine homozygot-null Tiere, welche kein aktives *B3gnt5* Genes tragen, gezüchtet werden. Das fehlende Gen führte zum Tod in einem sehr frühen embryonalen Stadium. Das zweite Manuskript schildert die Resultate, die während diesem Teil der Arbeit gewonnen wurden, und zeigt mögliche Erklärungen auf, weshalb diese Mutation so letal ist.

3. Project Aims

1. The *B3gnt1* null mouse

The primary phenotypic examinations in the *B3gnt1* null mouse in this study were to describe and characterize the defects relating to the null animals' inability to procreate. Additionally, there was a shift in the Mendelian distribution of the null allele in the offspring obtained from heterozygous matings. The possible link between these two observations due to the effects of imprinting was also considered. In initial studies we were able to show that null males were not able to father progeny with wildtype female counterparts. Consequently we investigated parameters surrounding male murine reproductive capacity. This included the following:

1. Morphology and motility of null sperm
2. Sperm number
3. Ability of males to plug estrous females
4. Number of sperm ejaculated, collected from plugged females
5. Sperm-egg interaction and the sperm acrosome reaction
6. sperm migration in the uterus following mating
7. tracking the null allele via genotyping at various embryonic stages to check for imprinting
8. time course studies in vitro with eggs from wildtype mothers and either null or wildtype sperm
9. Behavior studies with wildtype vs. null males with estrous females
10. Comparison of null male behavior towards estrous females and males chemically treated to disrupt their olfactory bulbs

2. The *B3gnt5* null mouse

In a second study we aimed to examine the role of the *B3gnt5* gene and the glycolipids β3GNT5 modifies. To this end we prepared a targeting construct which upon correct recombination disrupted the coding exon with a neomycin resistance selection marker. Four separate embryonic stem cell (ES) clones were obtained with the correct insertion. Two chimeric founder males were obtained which exhibited germ line transmission of the null allele. Heterozygote mating of these mice never gave rise to homozygous null offspring. The complete lack of the *B3gnt5* gene was lethal to the embryo. Embryos at various stages of development were genotyped with PCR in an effort to determine the exact time of death. This study included the following experiments:

1. Creation of a heterozygous ES cell line for injection into murine blastocysts
2. Creation of founder males with germ line transmission of the null allele
3. Crossing heterozygous animals for the production of a null line
4. Genotypic analysis of developing embryos to determine time of death
5. *in situ* studies with wildtype embryos to show early embryonic expression of *B3gnt5* mRNA
6. Quantitative PCR to assess the level of expression of *B3gnt5* in ES cells

1. Basic concepts in mammalian glycosylation

Glycosylation refers to the modification of macromolecules like proteins and lipids by adding glycans (poly- or oligosaccharide chains), which in the case of proteins is a posttranslational modification (PTM). Unlike phosphorylation, acetylation, or ubiquitination which link specific functional groups or proteins to a protein, the addition of glycans is highly diverse and introduces complexity to the protein which is unmatched by the other PTMs. Even though there are less than ten monosaccharide units used to create glycans in mammals, the variation they achieve is unsurpassed by any other biological modification. The quantity and individuality of glycan chains is afforded by their structural variation and the versatility of the glycosidic linkages which connect the monosaccharides.

The unique structure of the monosaccharides offers first insights to their diversity as polymers. Cyclic representations of the most common mammalian monosaccharides are depicted in figure 1 with the carbons numbered according to the chemical nomenclature. With the exception of xylose (Xyl) and sialic acid (Sia) they are six carbon sugars or hexoses. Consequently Xyl and Sia can participate in fewer or more linkages with other monosaccharides than the other hexoses, respectively. Chemically, the monosaccharides are aldose (–CH=O) or ketose (–C=O) units depending on the functional group they contain. The main mammalian monosaccharides considered here are all aldoses. Monosaccharides in their linear configurations have more than one chiral center and can be divided into two different forms. The D or L form describes the orientation of the asymmetric carbon farthest away from the aldehyde functional group. As shown in figure 2A, the D and L configurations are mirror images of one another. D and L can only describe differences inherent to monosaccharides in their linear conformation. These two forms represent non-superimposable molecules and are thus distinct carbohydrates. In nature, monosaccharides can be found in their D and L-forms, but the D-forms predominate in mammalian glycosylation reactions. Only fucose (Fuc) is used in its L-form. Finally, since monosaccharides can exist as rings (fig. 2A) the carbonyl carbon becomes an additional asymmetric center (fig. 2B). This carbon is termed anomeric and gives rise to α and β stereoisomers due to the two ways in which the hydroxyl group can bind to this carbon. Analogously to the D and L configurations, the hydroxyl groups are

mirror images of one another. They can either face the same (α) or opposite (β) side of the C-1 as depicted in figure 2A.

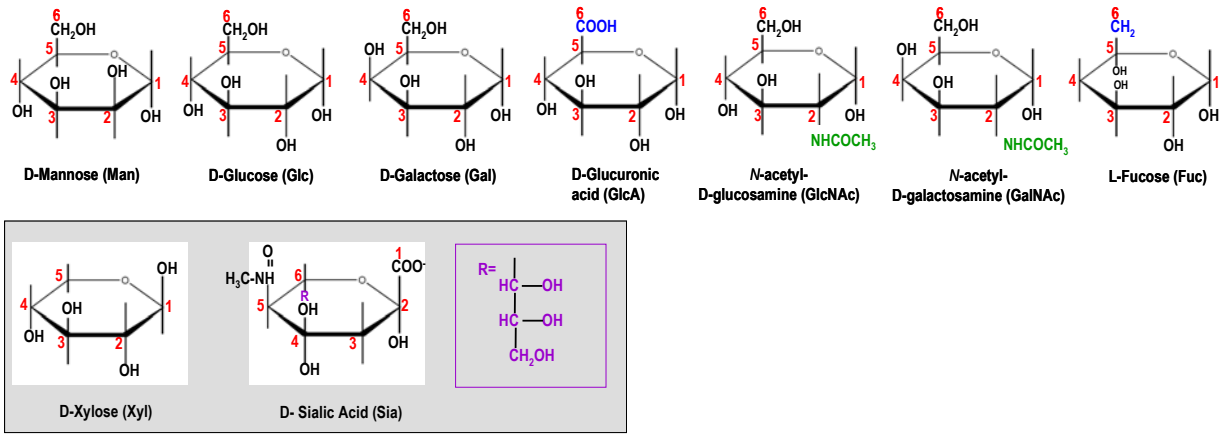

Figure 1: Mammalian monosaccharide structures and common abbreviations. Hayworth projections of the nine main monosaccharide units used to synthesize mammalian glycans.

The diversity in linkages between two sugars based on anomericity is one of the ways in which structure will relate to function and utilization of sugar molecules by mammals. A common example of this is the difference between the two glucose (Glc)- based polysaccharides cellulose $[Glc(\beta 1\text{-}4)Glc]_n$ and starch $[Glc(\alpha 1\text{-}4)Glc]_n$. Their different linkages render them completely different in their rigidity. While starch is a linear polymer made up of short Glc chains which are easily accessed as a quick energy source, cellulose polymers are long and dense making them very rigid and ideal for structural reinforcement. Moreover, while humans readily metabolize starch due to the availability of α-amylase, they cannot digest cellulose as a source of Glc because they lack β-glucosidase Not only can two sugars be connected in two unique ways and with several hydroxyl groups, but they can also branch. This means that one sugar moiety may bind up to five monosaccharides each of which can be extended independently leading to very complex and large structures (fig 2B).

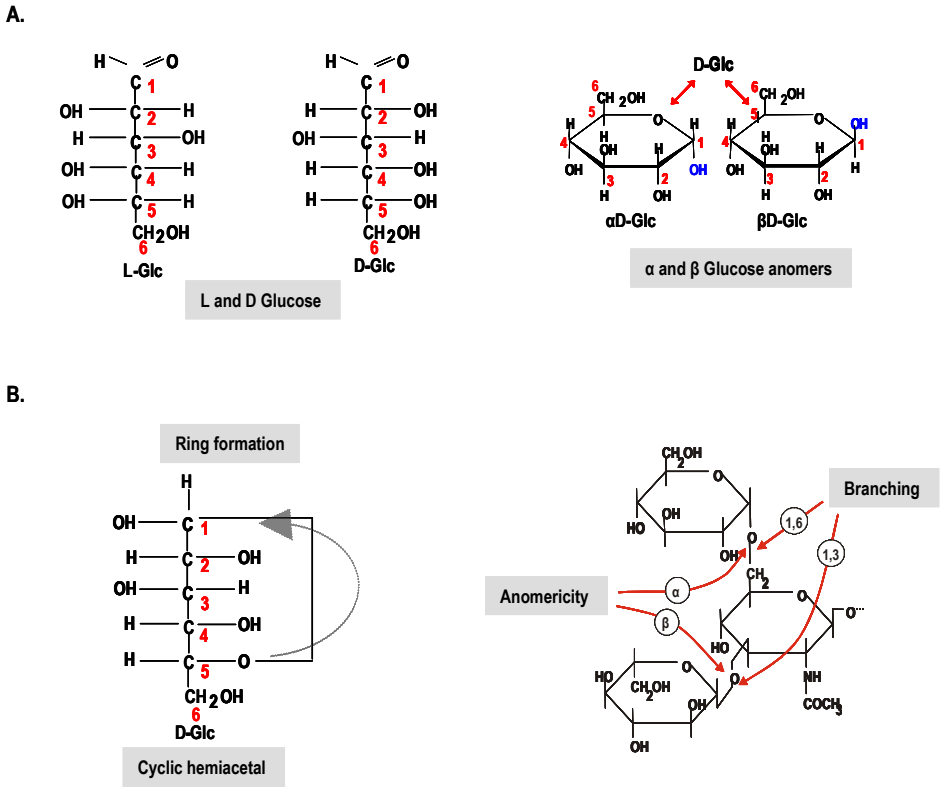

Figure 2: Properties of the monosaccharides (A) D and L configurations of Glc. The mirror images represented by these two configurations make them biologically distinct. Both D and L isomers can be further defined when they exist as cyclical carbohydrate structures. C-1 becomes an additional source of stereoisomerism and allows for the synthesis of either an α or β configuration of cyclic Glc. (B) Formation of a cyclic hemiacetal from a linear monosaccharide unit. The availability of α or β cyclic hemiacetals diversifies the way in which monosaccharide units can bind to one another. The orientation of the hydroxyl group on the C-1 chiral carbon dictates how one monosaccharide can link to another. The figure shows D-Glc with the hydroxyl group in such an orientation that it can synthesize either a 1,3 or a 1,6 linkage with another monosaccharide.

In addition to their intrinsic structures, glycans can be defined according to the molecules they modify into various classes of glycoconjugates. Currently there are seven classes of mammalian glycosylation described (fig 3). The most abundant is the N-glycan class (for review see [1, 2]). These are asparagine (Asn)- linked glycans found on proteins whose Asn-*N*-acetylglucosamine $(GlcNAc)_2$- Mannose $(Man)_9$-Glc_3 intermediate core is modified in the endoplasmic reticulum (ER) and consequently in the Golgi apparatus. The resulting Asn-$GlcNAc_2$ -Man_3 structure is then further modified in the Golgi apparatus. O-glycans are linked to a serine (Ser) or threonine (Thr) residue on proteins. Currently, six different monosaccharides have been implicated in the initiation of the O-glycans. The most common O-glycan is an extension of *N*-acetylgalactosamine (GalNAc)-Ser/Thr which is usually initiated in the cis-Golgi apparatus (for review see [3, 4]). The modification of Ser/Thr with Man or Fuc occurs in the ER [5, 6]. Glc residues can also be found attached to Ser/Thr. Even though this modification has been observed in human proteins such as Notch-1 and Factor VII, its functional significance and

biosynthetic location continues to elude researchers [7, 8]. Historically, it was believed that glycosylation was limited to the ER and the Golgi apparatus. However, nuclear and cytoplasmic glycosylation have been documented also [3, 9]. In fact, O-linked GlcNAc may be one of the most common glycosidic modifications found in cells and can be synthesized in the cytoplasm or nucleus. Finally, in collagen, hydroxylysine can act as a substrate for O-glycans. In this case, galactose (Gal) is added to hydroxylysine in the ER.

Glycans are not only used to directly decorate proteins, but can also be found attached to lipids to form glycolipids (for review see [10, 11]), or linked to phosphoinositol to create glycosylphosphoinositol (GPI) anchors (fig. 3) (for review see [12]). Glycolipids are initiated when Glc or Gal is added to ceramide (Cer) in the cis-Golgi compartment. Further elongation of this structure can continue in the Golgi apparatus. GPI anchors replace the C-terminus of the proteins they modify. The carboxyl-terminal signal initiates its replacement by cleaving itself from the protein allowing for substitution with the GPI. The GPI and provides an anchor point for these proteins into the membranes. GPI anchors are presassembled are pre-synthesized on the cytosolic face of the ER, while their transfer to proteins occurs on the luminal side of the ER [13]. Finally, glycosaminoglycan (GAG) chains are unbranched repeating units of disaccharides often found sulfated (for review see [14]). They attach covalently to proteoglycans in the ER or the cis-Golgi compartment. One of the simplest GAG chains is the disaccharide hyaluronic acid which can also be found in an unbound form in high concentrations in skin, skeletal tissue, and umbilical cord (for review see [15-17]). Contrary to what we know about glycan biosynthesis, hyaluronic acid is synthesized at the plasma membranerather than in the Golgi apparatus [13]. GAG chains are otherwise almost exclusively initiated and elongated in the Golgi apparatus. So far, the only ER-dependent step identified in GAG synthesis involves the addition of Xyl to its proteoglycan substrate [14, 18].

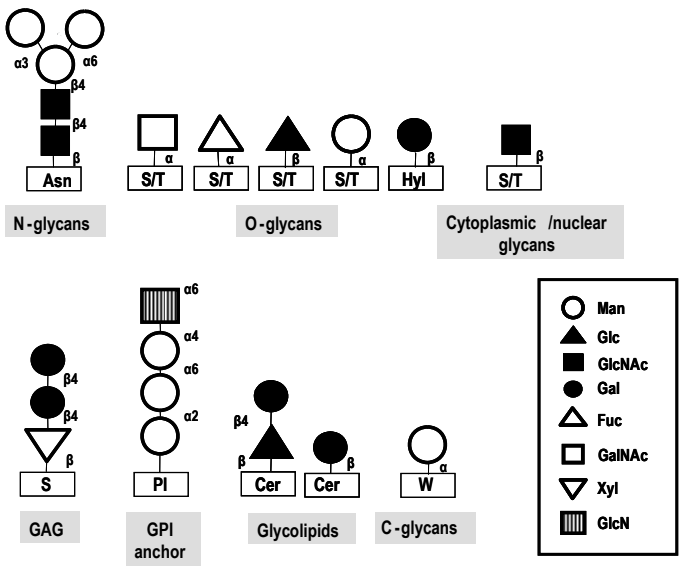

Figure 3: Seven known classes of mammalian glycosylation.

Generally glycans can be divided into two functional classes comprising physical or modulator functions, and secondly recognition or ligand functions. As structures, glycans can act as barriers or biological scaffolds. The extracellular matrix, for example, is largely made up of proteoglycans [14, 19] and collagen [20] which can alter the phenotypes of cells *in vivo* and *in vitro*. Without glycosylation, barriers between single cells and various tissues and organs would be very different as they would only have the properties afforded by proteins and lipids. Carbohydrate chains can bind water molecules very effectively. This interaction with water molecules forms gels which can act as a protective layer within an organism. In addition to being structural modifiers, glycans influence the tertiary structure of proteins such as nerve growth factor receptor or CD2 [3, 21-23]. Moreover, glycans can 'mask' otherwise immunogenic antigens or epitopes protecting pathogens from the host immune system [24]. For example, HIV has a heavily glycosylated protein (gp120) on its surface which has 24 N-glycosylation sites. These sugars make up more than 50% of its mass and serve to shield the virus from immune recognition and antibody attack [25, 26]. However, the protective functions of glycans do not only serve invading pathogens. High molecular weight glycoproteins like the mucins are protected from protease activity via their dense O-glycans in tandem repeat domains [27, 28]. Glycans also give mucins their effective function as coating layers protecting surfaces like the bladder [29] or the oviduct [30] from damage. Finally, O-glycosylation has been implicated in providing heat resistance to glycoproteins like the mucins [28, 31].

The role of glycans as ligands can be further subdivided into intracellular and intercellular or extracellular recognition and interactions. In their intracellular functions, glycans can act as signals or recognize intracellular ligands. One critical intracellular signaling event dependent on glycans is glycoprotein folding and the action of chaperones such as calreticulin and calnexin. These chaperones make use of a lectin-like interaction with glycan structures on N-glycosylated proteins which mark them for folding modifications. Calreticulin and calnexin will only interact with the monoglucosylated Man core [32-34] (fig. 4). Events surrounding intracellular trafficking can also be dependent on glycans for recognition interactions [35]. The Man-6-phosphate receptor is perhaps the most studied structure in intracellular trafficking. Man-6-phosphate marks proteins for sorting to the lysosomes [36, 37]. Endogenously controlled cell surface signals like desialylated Gal or GalNAc glycans have been shown to act as ligands for the asialoglycoprotein receptor and are used to mark molecules for clearance from circulation [38-40].

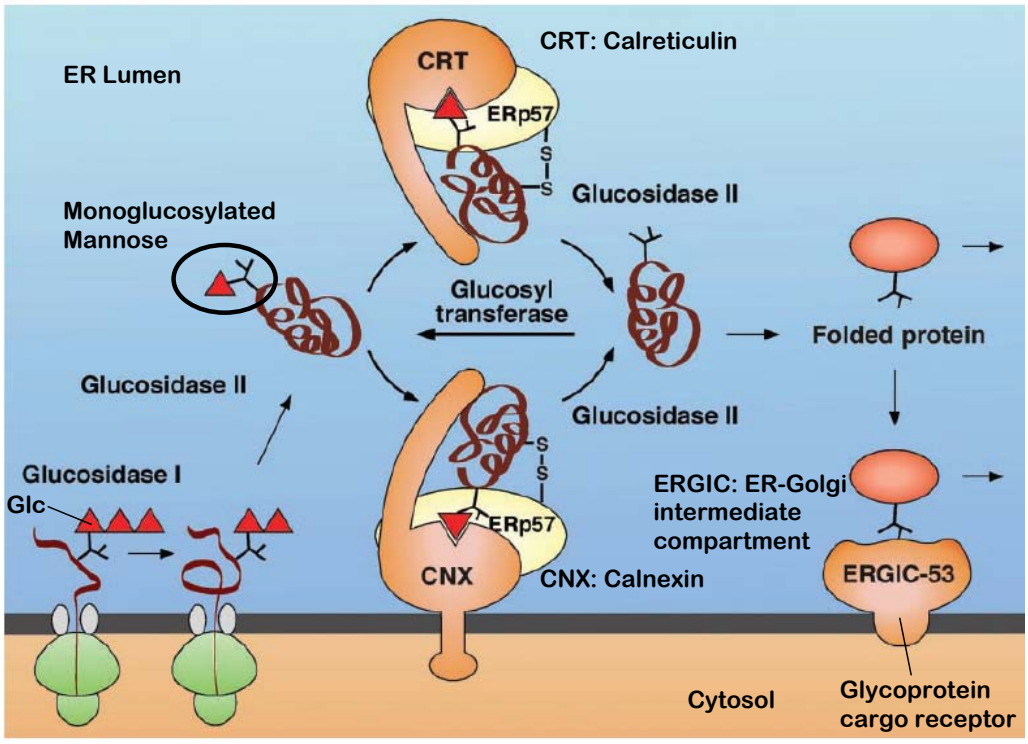

Figure 4: Interaction of chaperone molecules with glycans on the surface of glycoproteins. The monoglucosylated glycan acts as a ligand receptor for the chaperones Calreticulin (CRT) and Calnexin (CNX) in the ER. The glycoprotein then enters the ER-Golgi intermediate compartment (ERGIC) via ERGIC-53, a membrane lectin that acts as a cargo receptor for glycoproteins leaving the ER and entering ERGIC. (Adapted from [34])

Extracellular interactions involving glycans refers to communication with symbionts or invaders. Some pathogenic invaders try to avoid recognition by the immune system via molecular mimicry. In this context, mimicry refers to a pathogen blending with its host

surroundings by adopting or expressing similar glycan moieties to those found on host cell surfaces. This can have dire consequences when the host does recognize the invader as a threat and mounts an immune answer which also recognizes host glycans and leads to autoimmunity. One autoimmune disease which affects the peripheral nervous system is Guillain Barre Syndrome (GBS). GBS was first described by Guillain and his colleagues in 1916 before much was known about the molecular mechanisms used by the human immune system [41]. Later, it was observed that some patients suffering from the disease were often infected with the *Campylobacter jejuni* bacteria preceding the onset of GBS [42]. Experiments showed that molecular mimicry of *C. jejuni's* lipooligosaccharides (LOS) to GM1 gangliosides, a glycolipid, expressed in the nervous system (fig. 5). This causes the immune system to produce IgG antibodies against this specific glycan structure [42-44]. As a result the gangliosides in the nerves are attacked by the host immune system causing nerve inflammation, demyelination, and eventually paralysis.

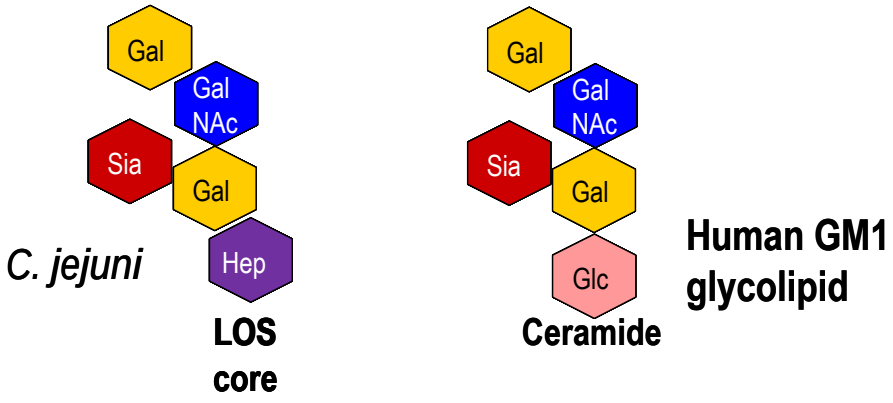

Figure 5: Molecular mimicry. The LOS chains on *C.jejuni* mimic those of the GM1 glycolipids found in the peripheral nervous system. The similarity of these two structures elicits an immune response causing GBS. (Hep: heptose)

Alternatively, carbohydrates on host cell surfaces may be utilized as binding sites for certain pathogens. Many viruses use the carbohydrates expressed on cell surfaces as viral entry receptors. Often, these entry receptors are negatively charged GAGs or heavily sialylated glycans [45]. Influenza viruses have been shown to recognize endogenous Sia [46, 47]. Avian influenza A infects cells carrying the α2,3 Sia which it uses as a ligand [48-50]. Human influenza A has been shown to bind α2,6 linked Sia which is abundantly available in the human respiratory tract [49] making this an ideal site for infection. The influenza viruses use a hemagglutinin (HA) protein receptor to bind these specific Sia ligands. The genome of the influenza virus mutates frequently allowing them to alter

their HAs specificities. The resulting mutated HA protein effectively binds a new receptor. This allows the viruses very efficient adaptation to new ligands and is believed to be responsible for interspecies crosses causing past global flu pandemics [51]. Genomic analysis of the HA5 subunit in avian Influenza A shows that changes in just a few amino acids can alter its specificity from α2,3 Sia to α2,6 Sia [52, 53] rendering it pathogenic to human cells. Finally, cell surface glycans can be used by other viruses as recognition or entry receptors. It is an interaction with host cells that is available viral pathogens including the rotaviruses, human respiratory syncytial virus, and the noroviruses [54-56].

2. Structure, function, and nomenclature of eukaryotic glycosyltransferases

Glycosyltransferases (GTs) catalyze the formation of glycosidic linkages needed to connect monosaccharides together or to their substrates. GTs assume different structures depending on their intracellular location which include the nucleus, the ER, the cytosol, or the Golgi apparatus; the latter being where both the enzymes examined in this study are localized. GTs generally add monosaccharides provided by high energy nucleotide sugars to an acceptor molecule; i.e. another sugar or a biological substrate as shown in figure 6. These high energy nucleotide sugars are the products of phosphorylated sugars and nucleotide triphosphates (NTP) like uridine triphosphate (UTP). As a result of the reaction, UTP loses a phosphate leading to UDP-monosaccharides which can be used in glycoconjugate biosynthesis. Nucleotide sugars are transferred to the ER or Golgi apparatus via nucleotide sugar transporters which are electroneutral antiports (fig. 6).

GTs are commonly defined according to their donor and acceptor substrates. For example, GTs that catalyze the attachment of a βGlcNAc residue to another monosaccharide is termed a βGlcNAc-transferase. Additionally, the carbon of the acceptor and hydroxyl group of the donor sugar occupied by the glycosidic linkage are used to categorize GTs. Hence, a β1,4 GT catalyzes the linkage of the β anomeric carbon of the donor monosaccharide to the C-4 hydroxyl group of the acceptor glycan. GTs that catalyze the same linkage between two sugars are grouped into functional families. Different members of the same family are numbered, i.e. β1,3GlcNAc-transferase -1, -2, -3, etc. GTs with the same catalytic donor and acceptor glycans often differ in the biological substrates they act on (protein, lipid, GAG, etc). Different family members may also be defined when the GTs are simply expressed in an organ- and/or developmentally-specific manner [57].

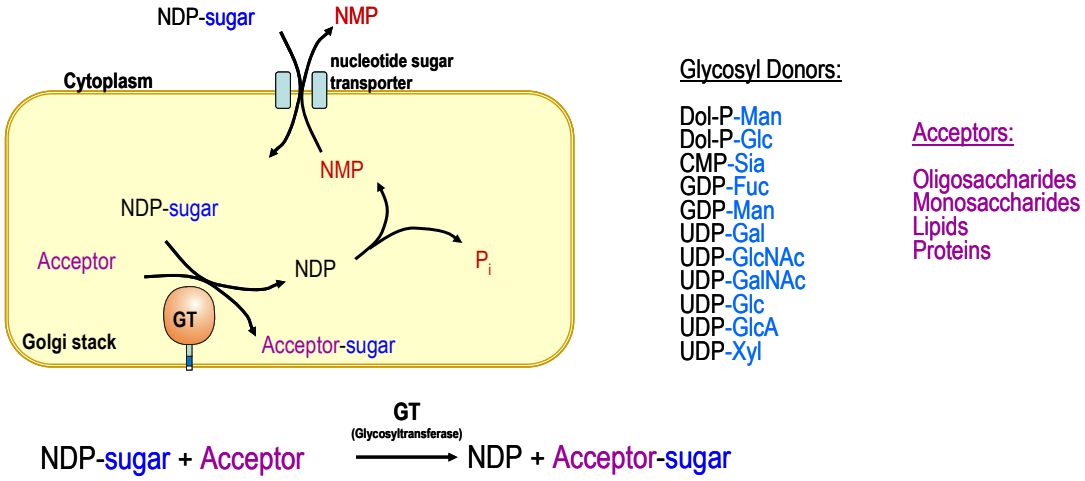

Figure 6: Subcellular localization of GT reactions: The monosaccharide donors are shuttled into the Golgi via nucleotide sugar transporters. The GTs which are anchored into the Golgi apparatus membrane catalyze the addition of monosaccharides from donor nucleotide sugars to their acceptor substrates. The monosaccharide is released from its nucleotide sugar donor, and one of its available hydroxyl groups is transferred to an available carbon acceptor which will be dictated by the specificity of the GT. The resulting linkage is hence dependent on both the specificity of the GT and the availability of donors and acceptors.

In keeping with their functional diversity, GTs exhibit relatively high variation in their primary sequences. The classification of GTs has undergone multiple adaptations to keep up with the newest information about their constituents. Today, the **C**arbohydrate-**A**ctive en**Zy**mes (CAZy) classification predominates (for review see [58, 59]). In this system, the GT families are grouped according to both their structural and catalytic properties as described above. The Golgi GTs share significant secondary structural homologies. They are type II transmembrane proteins with a single transmembrane domain. This domain is flanked by a short cytosolic amino-terminal end and a larger carboxy-terminal part in the lumen of the Golgi apparatus (fig. 7). This part contains a stem-region to which the actual catalytic domain is attached an in turn gives the enzyme its specificity. ER and Golgi GTs are both anchored to the membranes of these organelles. The nuclear and cytosolic GTs are soluble and bear some similarity to one another in their secondary structures [60]. Although the activity of certain cytosolic GTs have been described [61-63], only one of these mammalian GT's has been shown to also localize to the nucleus; an O-GlcNAc transferase [62, 64, 65]. However, like their ER and Golgi counterparts, little homology is found among these soluble GTs when their primary sequences are compared.

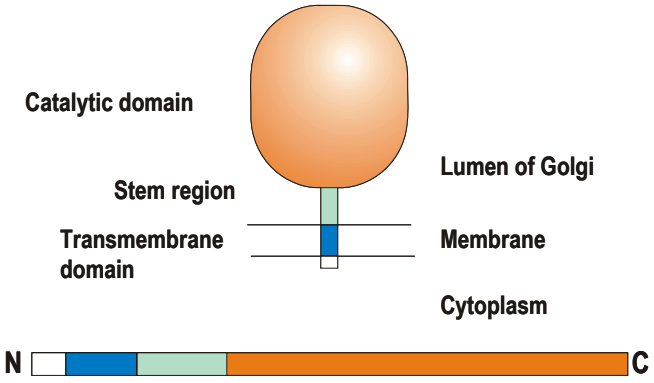

Figure 7: Basic structure of a Golgi GT. The GT is anchored to the Golgi membrane via its transmembrane domain. The catalytic site faces the lumen of the Golgi, while only a small portion of the N-terminus is in the cytoplasm.

The nomenclature described above cannot apply to GTs initiating a glycan on a biological substrate. These special GTs are often named for the glycan products they initiate, i.e. glucosylceramide synthase or the different Ser/Thr modifying GTs in O-glycosylation. In contrast to classical elongating GTs in the Golgi apparatus, the donor substrates may be nucleotide sugars, lipid activated monosaccharides, or preassembled lipid linked glycans. For a more complete review of GTs initiating glycolipids, O-glycans, GPI anchors, and N-glycans the reader is referred to references [3, 11, 12, 66].

Investigations have shown that linkage types (i.e. β1,3 or α2,3) commits to structural conservations among different GTs. Consequently, sequence homology among these GTs in their catalytic domains is not uncommon. As a result a β1,3Gal- transferase shares more homology with a β1,3GlcNAc- transferase than with a β1,4Gal- transferase [67]. This suggests that there might be genetically similar sequences particular to a specific linkage between two monosaccharides (i.e. β1,3). However, the monosaccharides involved may vary in their donor or acceptor roles. Sia and Fuc transferases are exceptions since these enzymes display high sequence homologies even when catalyzing different linkages.

3. The β1,3GlcNAc-transferase family

The β1,3GlcNAc-transferase (*B3gnt*) family is made up of genes which encode GTs that catalyze the addition of β1,3GlcNAc onto a Gal or GalNAc. The mammalian *B3gnt* gene family currently has 5 members with confirmed functions and one (the *ignt*) whose protein function is under debate. All known *B3gnt* genes and the proteins they code for are listed in Table 1 along with their chromosome locations, acceptor and substrate specificities, and tissue expression profiles. All the enzymes encoded for in this family except β3GNT6 add a GlcNAc to a Gal leading to the (GlcNAc β1,3- Gal β1,4) product. This product is termed a lactosamine or a polylactosamine when multiple units are linked together on a substrate. β3GNT6 initiates core 3 O-glycosylation by adding a β1,3GlcNAc to a an O-GalNAc.. As noted in Table 1, lactosamines are found on all classes of glycoconjugates. Those enzymes listed in bold are the focus of this study.

Table 1: *B3gnt* Gene Family

Gene (Protein) Name	Chromosome (murine/ human)	Acceptor Glycan	Substrates	Expression Profile
ignt (iGNT)		Gal	N-/O-Glycans	Fetal brain, all adult tissues
B3gnt1/2[1] **(β3GNT1)**	**11/2**	**Gal**	**N-/O-Glycans, Glycolipids, GAG**	**All adult tissues**
B3gnt3 (β3GNT3)	8/19	Gal	O-Glycans Core 1 elongation	Colon, placenta, stomach, jejunum, and HEVs
B3gnt4 (β3GNT4)	5/12	Gal	ND	Brain
B3gnt5 **(β3GNT5)**	**16/21**	**Gal**	**Lactosylceramide Glycolipids**	**Placenta, testes, spleen, embryo**
B3gnt6 (β3GNT6)	7/11	GalNAc	Core-3 O-Glycans	Mucins in stomach, colon, and small intestine

[1] The same gene/enzyme is described in both studies under different names. Here the enzyme will be called *B3gnt1* and the protein β3GNT1.

The iGNT was the first enzyme with lactosamine synthase activity described [68]. It was found to be especially active on blood group antigens. Its name derives from the embryonic i-antigen, which changes to its adult form, the I-antigen, later in development

(fig. 8). Although the gene described has a unique sequence, the lactosamine or polylactosamine synthase activity ascribed to iGNT could not be substantiated when tested in other labs, including our own.

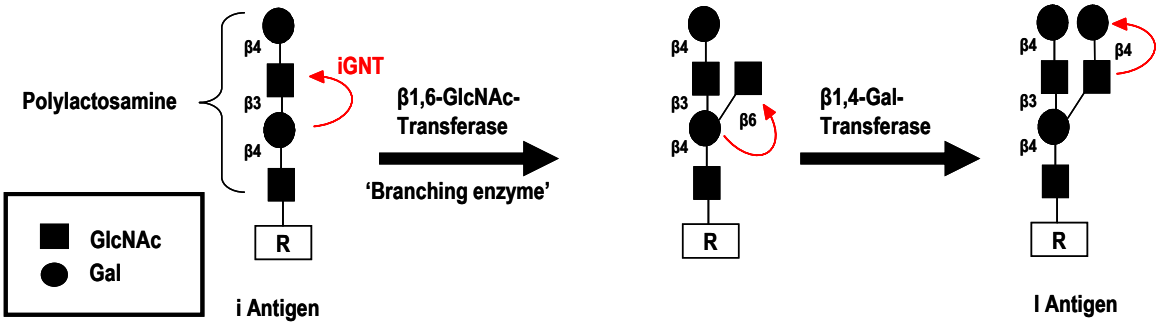

Figure 8: Synthesis of the i and I-antigens

β3GNT1 is the enzyme with the broadest substrate specificity and tissue expression profile discovered thus far [69, 70] (fig. 9). This Golgi enzyme not only elongates glycans, but it has been shown to initiate and elongate polylactosamines in particular [69]. As part of this study we obtained mice with a targeted disruption of the *B3gnt1* gene. Our phenotyping experiments showed that this gene regulated lactosamines or polylactosamines on various biological structures including parts of the brain and T-cells. The polylactosamines have previously been shown to regulate T-cell activity [71] and developmental antigen regulation on blood cells [72]. In addition, lactosamine structures under the control of β3GNT1 have been implicated in neuronal processes such as axon guidance in the olfactory epithelium (OE) and vomeronasal organ (VNO) [73] and play a role in directing the migration of gonadotropin-releasing hormone (GnRH)-neurons in the brain [74].

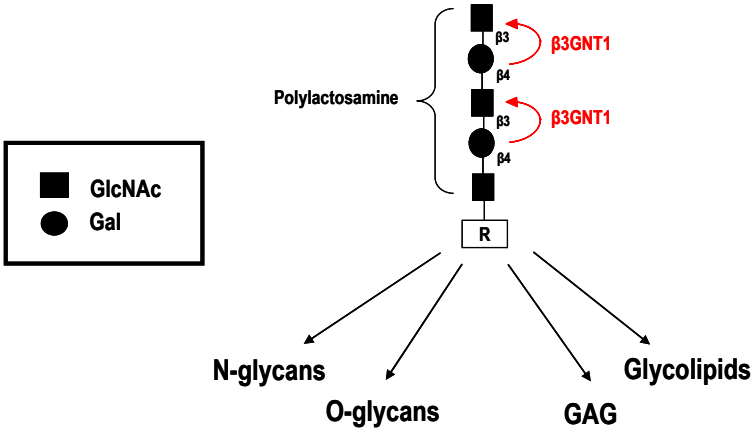

Figure 9: β3GNT1 elongates lactosamine chains on various glycan substrates. B3GNT1 is the rate limiting enzyme in the formation of polylactosamine chains. These chains can be found on N and O-glycans, on GAG chains, and on glycolipids.

Little is known about the activity of the β3GNT3 and β3GNT4 enzymes. Both were discovered in a study in which cDNA from human gastric mucosa and from the neuroblastoma cell line SK-N-MC were screened for *B3gnt1* homologs [70]. Both were shown to contain shared motifs found in members of both the *B3galt* family and the *B3gnt* family. They are distinguishable from β3GNT1 by their substrate specificities which for β3GNT3 includes core-1 O-glycosylation elongation. Recombinant expression studies revealed that β3GNT3 and 4 had the same monosaccharide substrate acceptor as β3GNT1, namely Gal, but that the complete substrate structure was unique. As shown in Table 2, β3GNT3 has high activity with lactose and lacto-N-tetraose and some activity on *p*-lacto-N-neohexose. β3GNT4 shows only limited activity on lacto-N-neotetraose. In addition to structural preferences, the expression profiles of β3GNT1, 3, and 4 are different. While β3GNT1 is ubiquitously expressed, β3GNT3 was detected mostly in the colon, placenta, stomach, jejunum, and high endothelial venules. β3GNT4 expression is limited to relatively low levels in the brain.

A recent study with the core-1 elongating enzyme β3GNT3 has provided new information about the role of this enzyme *in vivo* [75]. Mice with targeted null mutations for *B3gnt3* were examined for their lymphocyte homing capabilities. The core-1 O-glycans serve as scaffolds for the MECA-79 antigen whose synthesis was suspected to be under the control of the β3GNT3 enzyme. This study showed that β3GNT3 is solely responsible for the core-1 extension necessary for MECA-79 expression which binds L-selectin. The loss of MECA-79 corresponded with higher in vivo rolling velocities of B and T lymphocytes due to this lost interaction with L-selectin. Moreover, there were decreased numbers of B lymphocytes in the *B3gnt3* null animals although the total number of lymphocytes was compensated for with increased T cell populations [75].

Table 2: Preferred substrates for β3GNT1, 2, and 3. (Adapted from [70].)

Substrate	Structure	β3GNT1	β3GNT3	β3GNT4
Lacto-*N*-neotetraose (LNnT)	**Gal**β1-4GlcNAc β1-3Galβ1-4Glc	100[a]	100	100
Lactose	**Gal**β1-4Glc	67	235	33
Lactosamine	**Gal**β1-4GlcNAc	96	0	4
Lacto-*N*-tetraose	**Gal**β1-3GlcNAcβ1-3Galβ1-4Glc	6	114	0
p-lacto-*N*-neohexose	**Gal**β1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-Galβ1-4Glc	ND	45	0

[a] Activities are expressed as a percentage of the activity for pyridylaminated (a fluorescent labeling for sugar chains using 2-aminopyridine) LNnT .

β3GNT5 represents a distinct member of the β3GNT family which is responsible for initiating lacto-series (Lc3) glycolipids (fig. 10) [76, 77]. The Lac-Cer glycolipid core serves as a substrate for numerous glycolipid structures. Among others, the lacto-series (GlcNAc β1-3- Gal β1-4) unit acts as a scaffold for the synthesis of blood group antigens. These are essential for the formation of the A, B, and O antigens and some of the stage specific embryonic antigens (SSEA) [66]. β3GNT5, also a Golgi GT, was found to be highly expressed in adult murine spleen, placenta, and to some degree in the testes [76]. *In situ* analyses revealed broader expression of the enzyme during embryonic development compared to its adult profile [76] including the central nervous system, spinal cord, heart, intestines, and retina. Studies using zebrafish and xenopus have

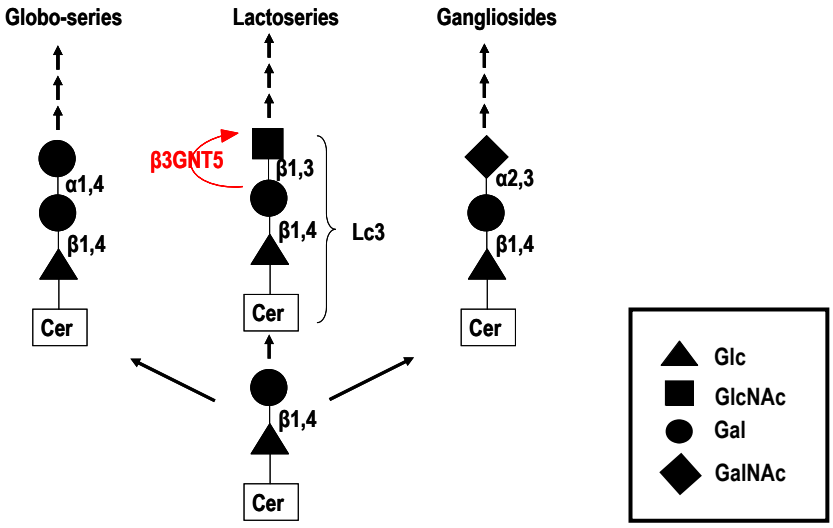

Figure 10: Overview of glucosylceramide glycolipid biosynthesis. β3GNT5 catalyzes the initiating step for the production of lacto series glycolipids. The Lac-Cer structure is the basis for all of these glycolipids.

identified an ortholog for Lc3 synthase gene [78, 79]. Both studies showed that *B3gnt5* was expressed during embryonic development. Notably, the gene seems to be important for lens development in the zebrafish. This was similar to findings in the mouse where it was also found in the developing eye [76]. An *in vivo* analysis of *B3gnt5* in the mouse comprises the second part of this study. We targeted the *B3gnt5* gene by introducing a neomycin resistance cassette into the single coding exon of *B3gnt5* in order disrupt its transcription, thus enabling the investigation of *B3gnt5's* physiological role of this gene and its products *in vivo*.

β3GNT6 activity had, until recently, only been described for the human enzyme [80, 81] although a predicted murine β3GNT6 ortholog is listed in GenBank. Human β3GNT6 is the enzyme that elongates core-3 O-glycans [80]. Core-3 O-glycans are the result of a β1,3GlcNAc addition to a GalNAc (β1,0) -Ser/Thr (fig. 3). Core-3 O-glycans (figure 11) are structural precursors of more than half of the mucins found in all parts of the intestines [82]. Quantitative PCR verified high expression in the stomach, colon, and small intestine, all areas in which mucins are found in high concentrations. Additional studies showed that β3GNT6 was down-regulated in gastro- and colorectal cancers [81]. Furthermore, expression of β3GNT6 in tumor cells injected into nude mice was able to lower the metastatic migration of tumor cells [81]. Glycoepitopes have long been studied as possible cancer markers [11, 83]; the core-3 O-glycans offers just one example of how glycans can modulate cancer cells and their effects on the host. Recently, the disruption of *B3gnt6* showed that this gene does code for the protein responsible for core-3 O-glycan elongation in mice by *An et al. (2007)* [84]. In addition the authors were able to confirm the importance β3GNT6 modified core-3 O-glycans in preventing susceptibility to colorectal tumors and colitis in the murine model. This parallels findings observed *in vitro* with the human ortholog.

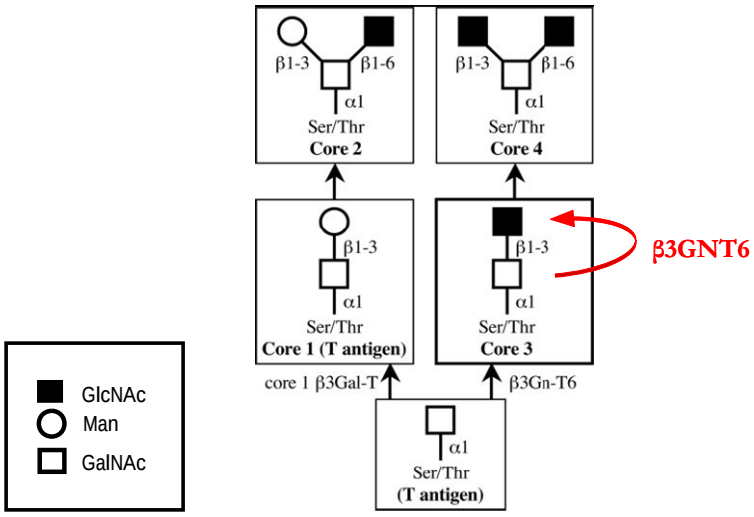

Figure 11: Core 3 biosynthesis of O-glycans (taken from [81]). The activity of β3GNT6 is limited to O-glycans. Here β3GNT6 is adding the GlcNAc to a GalNAc acceptor rather than to a Gal. This is the only family member with an acceptor other than Gal.

The *B3gnt* gene family encodes enzymes involved in the modifying Gal or GalNAc with β1,3GlcNAc. The lactosamines resulting from the activities of β3GNT1-5 have been detected on all the known glycoconjugates classes. Both these substrates and the activity of the *B3gnt* family of genes can fall under developmental, expression, or tissue specific control mechanisms. Since the catalytic activities and donor- acceptor substrate specificities are so similar for these enzymes, the best way to study them individually is to disrupt them one by one *in vivo*. The availability of murine embryonic stem cells provides a mammalian model for the analysis of these genes and their products by inactivating their coding regions via insertion or deletion cassettes. Results obtained will give more insight into the unique organ, developmental, and substrate specificity for each family member. We aim to detect phenotypic abnormalities resulting from the inactivated gene that can give us more information about when, where, and with what acceptor substrates the β3GNT enzymes interact.

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Manuscript 1:

This manuscript describes the results obtained from investigations into the male fertility phenotype observed in the B3gnt1 null male mice. It was recently accepted for publication (July 2007) in the peer reviewed journal *Molecular Reproduction and Development.*

IMPAIRED SEXUAL BEHAVIOR IN MALE MICE DEFICIENT FOR THE β1-3 N-ACETYLGLUCOSAMINYLTRANSFERASE-I GENE

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ABSTRACT

The β1-3 *N*-acetylglucosaminyltransferase-1 (*B3gnt1*) gene encodes a poly-N-acetyllactosamine synthase which can initiate and extend poly-N-acetyllactosamine chains [Gal(β1-4)GlcNAc(β1-3)$_n$]. Previous investigations with heterozygous and homozygous null mice for this gene have revealed the importance of poly-N-acetyllactosamine chains for the formation of olfactory axon connections with the olfactory bulb and the migration of gonadotropin releasing hormone neurons to the hypothalamus. The possible long term effects of these developmental defects, however, has not yet been studied. Here we have examined a reproductive phenotype observed in *B3gnt1*-null mice. Whereas the *B3gnt1* null females were fertile, the *B3gnt1* null males were not able to sire litters at the expected rate when mated to either wildtype or *B3gnt1*-null females. We assessed male sexual behavior as well as male reproduction parameters such as testes size, spermatogenesis, sperm number, morphology, and the development of early embryos in order to identify the source of a reduced rate of reproduction. Our findings show that the *B3gnt1* null male reproductive organs were functional and could not account for the lower rate at which they produced offspring with wildtype conspecifics. Hence, we propose that the phenotype observed resulted from an impaired sexual response to female mating partners.

Running head

Sexual deficiency of male *B3gnt1*-null mice

Keywords

Glycosylation, poly-N-acetyllactosamine, gene targeting, olfaction

INTRODUCTION

Olfaction is important in modulating various behavioral responses in mammals (Bakker 2003). Olfactory cues influence social interactions, such as aggression, suckling and mating behavior. While it was commonly accepted that these innate behaviors are largely mediated by pheromones detected by neurons in the vomeronasal organ (VNO) (Wang et al. 2006), recent studies have shown that the main olfactory epithelium (MOE) may in fact be involved in the processing of pheromone or other odorant signals relevant for sexual behavior (Keller et al. 2006; Keverne 2004; Yoon et al. 2005). Specific defects in the MOE exclusive of the VNO are responsible for impaired reproductive behavior in males, due to their inability to recognize estrous females and manifesting in decreased mounting behavior (Yoon et al. 2005).

Poly-N-acetyllactosamine chains are involved in axon pathfinding of olfactory sensory neurons from the MOE to the glomeruli in the main olfactory bulb (MOB) (Henion et al. 2005). The β1-3 N-acetylglucosaminyltransferase-1 (*B3gnt1*) enzyme adds β1-3 linked N-acetylglucosamine to galactose, thereby enabling the formation of poly-N-acetyllactosamine chains (Zhou et al. 1999). In mammals, more than 200 glycosyltransferase enzymes control the covalent bond formation of carbohydrate donors to acceptor molecules via specific linkages (Varki and Marth 1995). *B3gnt1* represents one form of five known genes belonging to the *β3gnt* family. Poly-N-acetyllactosamine chains are found in N- and O-glycan chains, in glycolipids, and in keratin sulfate glycosaminoglycans. This broad distribution may account for poly-N-acetyllactosamines being implicated in many different biological processes. Initially, the presence of these glycan chains was associated with the metastatic potential of cancer cells (Dennis et al. 1987). Physiologically, poly-N-acetyllactosamines were

implicated in T-cell proliferation by mediating the clustering of T-cell proteins with the galectin lattice (Demetriou et al. 2001) and by regulating T_{H1} cytokine production (Morgan et al. 2004).

In addition to its role in axon guidance to the MOB, the *B3gnt1* gene has been shown to be expressed by embryonic day 13 in parts of the VNO (Henion et al. 2005). This coincides with the time point at which gonadotropin releasing hormone (GnRH) neurons are observed migrating from the VNO to the forebrain in concert with *B3gnt1* modulated poly-N-acetyllactosamine structures (Bless et al. 2006). The expression of the specific glycans modified by the *B3gnt1* enzyme in the MOE and along the VNO towards the hypothalamus, together with its role in axon and neuronal guidance in these areas, suggested a possible involvement of the *B3gnt1* gene in reproductive behaviors and gametogenesis. To evaluate this hypothesis, we have examined these processes in *B3gnt1* null mice.

MATERIALS AND METHODS

Mouse Breeding and genotyping - *B3gnt1* null mice were established as previously described (Mitchell et al. 2001). The *B3gnt1* null allele has been backcrossed to the C57Black/6 background and to the NMRI outbred background for at least 6 generations. Animals were housed under a 12 h light-dark cycle with light beginning at 6 am. Breeding and experiments were performed in compliance with the Swiss Animal Protection Ordinance. PCR was used to genotype offspring. A 700 bp fragment was amplified from the wildtype *B3gnt1* allele (5'-CCT GAT GAT GCC AAA TGT CTT C-3' and 5'-ATC ATC GCC CTT GAA GAC AAA C-3') and a 1071 bp fragment was amplified from the *B3gnt1* null allele (5'-TCC GGA CAT GCT TAA GTT TGA G- 3' and 5'-AAC ATA AAG TGA CCC TCC CCA C-3'). Alternative primers were used to genotype blastocysts. For the wildtype allele a 637 bp fragment was amplified (5'-GCT GAA CAG GTG GTA CAA-3' and 5'-TGT GGA TCA CGT CAC CTA-3'). A 326 bp fragment of the geo cassette was amplified for the *B3gnt1* null allele (5'-CAT CAG CCG CTA CAG TCA AC-3' and 5'-CAT CAG AGC AGC CGA TTG TC-3').

Flow cytometry - Thymocytes (0.1 x 10^6 cells/100 μl) from 8-12 week old mice were labeled with FITC-conjugated lectins Lycopersicon esculentum agglutinin (LEA), concanavalin-A (ConA), and Artocarpus intergrifolia (Jacalin) (all from Vector Laboratories, Burlington, CA, USA) at 1 μg/ml, 2.5 μg/ml, and 0.5 μl/ml, respectively, for 15 min on ice. Cells were washed by centrifugation and resuspended in 1 ml of phosphate buffered saline. Cells were analyzed with a FACScan flow cytometer (BD Biosciences) using the CellQuest software.

Sexual behavior – Wildtype and *B3gnt1* null males were tested for their response to hormone-induced estrous wildtype females as described (Yoon et al. 2005). Briefly,

trials were done with 2-4 month old wildtype and *B3gnt1* null males. The males were each housed with a wildtype female for 5-7 days prior to testing. The females were removed from the males' cages 3 h prior to the experiments, which were started 1 h after the beginning of the dark cycle (7pm) of the mouse housing facility. A sexually receptive female was placed in the respective male cages, and the males' response to this female in terms of sniffing and mounting was observed and documented for 20 minutes. The observation was repeated for each male with another sexually receptive female with two different blind observers. In a second test we investigated the specific role of the MOE in sexual response to estrous females. Here we used sexually naïve wildtype males and were either injected with 2,6-dichlorobenzonitrile (25 μg/g body weight) in DMSO (2 μl/g body weight) or DMSO alone intraperitoneally on day 0, 2, and 4 . The2,6-dichlorobenzonitrile disrupts the MOE, while the DMSO alone provides mock injected control males. Experiments were run on the evening of day 7.

Histology - Testes were taken from wildtype and *B3gnt1* null mice and fixed in Bouin's solution. Testes were embedded in paraffin and cut into 3 μm sections. The sections were de-paraffinized for 2 washes in xylene for 10 min followed by sequential double washes in ethanol 100%, 96%, 80%, 70%, and in H_2O for 2 min each. Slides were stained with hematoxylin and eosin. Sections were viewed and photographed using Axio Vision software for the Zeiss Axiovet 200M microscope (Carl Zeiss AG, Feldbach, Switzerland)

Sperm isolation and analysis - Sperm were released from wildtype and *B3gnt1* null caudal epididymis into human tubal fluid (HTF) (Quinn et al. 1985) medium and counted. Sperm were tested for their ability to undergo the acrosome reaction by the Coomassie Blue method (Jeffs et al. 2001) using the calcium ionophore A23187 (Sigma) as a positive control to trigger the acrosome reaction. The sperm surface proteins ADAMs-2, -3 and PH-20 were detected by Western blotting using

antibodies against murine ADAMs-2, -3 (Chemicon) and PH-20 (a gift from P. Primakoff, University of Davis CA). Sperm were lysed in 3% SDS buffer (Cho et al. 2000), and 40 μg of lysate was loaded onto 10% SDS-PAGE gels and blotted onto nylon membranes for probing. Antibodies to ADAMs-2,-3 and PH-20 were diluted 1:1000, 1:2000, and 1:250, respectively.

Egg binding – Wildtype females were superovulated by intraperitoneal administration of 50 IU pregnant mare serum gonadotropin (PMSG) (Intervet, Veterinaria AG Zürich, Switzerland) and 25 IU human chorionic gonadotropin (hCG) (Intervet, Veterinaria AG Zürich, Switzerland) 46-48 h later (Hoogan et al. 1994). On the following morning, the females were sacrificed and egg clutches were removed from the oviducts. Egg clutches were released into pre-warmed (37 °C) HTF media with 30 μg/ml BSA, and fresh caudal sperm were added (1-2 x 10^6 sperm/ml). The sperm-egg mixture was observed at 10 min intervals under the microscope to monitor sperm hyaluronidase activity and the ability of sperm to interact with the zona pellucida of the eggs.

Sperm ejaculation – Isolated wildtype and *B3gnt1* null males were caged with superovulated wildtype females after the beginning of the dark cycle and approximately 12 h after the addition of 25 IU hCG to make sure the wildtype females were in estrus. Females were examined for vaginal plugs every 20 min. Plugged female were immediately sacrificed and their uteri removed. The uterine horns were carefully flushed with HTF medium to collect and count the sperm ejaculated into the uterus (Yeung et al. 2000).

Two cell stage transition – The development of zygotes up to the two-cell stage was investigated using the method of Krishna *et al.* (Krishna and Generoso 1977). Briefly, females were treated with PMSG and hCG as described above and caged with

wildtype and *B3gnt1* null males exactly 13 h after the hCG injection, *i.e.* 5-7 h into the dark cycle. Females were checked for the presence of a vaginal plug every 20 minutes, and the time of each plug was noted, which allowed for a time course for the development of embryos to the 2-cell stage to be documented. Plugged females were sacrificed and fertilized eggs removed in M2 media (Sigma). Fertilized eggs were then cultured under oil in M16 media (Sigma) at 37 °C and 5% CO_2.

Statistical analysis - Standard statistical tests were applied to data collected. For the distribution of the *B3gnt1* null allele and the comparison of male reproductive rates, the chi squared test was used. For data with more than one parameter, the 2-way ANOVA with repeated measures test was used. For single parameter tests, a one-way ANOVA with repeated measures was used.

RESULTS

The loss of poly-N-acetyllactosamine chains in *B3gnt1* null mice was confirmed using the poly-N-acetyllactosamine binding LEA lectin. Examination of thymocytes, a cell type expressing the *B3gnt1* gene at high levels, showed a major reduction of the LEA lectin staining and normal levels of ConA and Jacalin staining (**Fig. 1**). ConA binds to mannosylated structures, such as the core of N-glycans and Jacalin binds to the core1 of O-glycans. The normal ConA and Jacalin stains showed that the loss of poly-N-acetyllactosamine chains was not related to a decreased expression of N- or O-glycans on thymocytes.

Mice homozygous for a disrupted *B3gnt1* allele (Mitchell et al. 2001) did not display any altered viability as assessed by body weight and average lifespan. Breeding the *B3gnt1* null mice revealed an impaired reproductive capacity of the *B3gnt1* null males. *B3gnt1* null males almost never produced offspring when mated to females (**Table 1**). Each male was tested with one sexually receptive wildtype female per week over several months. Since wildtype females enter estrus every 5 days, a wildtype fertile male should sire 3-4 litters per month. Null males exhibit a much lower rate of successful sexual reproduction.

For the first behavioral test, males were housed with females for 5-7 days prior to the experiments to train them equally. Notably, some of the females housed with the wildtype males gave rise to viable litters while none of the wildtype females housed with the null males produced offspring as a result of this trial. The mating behavior was analyzed by scoring the amount of time males spent smelling females and the mounting attempts males made in repeated 20 minute trials. Males were tested with different wildtype females partners, which were all hormone treated to ensure estrus status, and hence promote male sexual interest. Compared to wildtype males, *B3gnt1*

null males showed little to no interest in estrous females ($p<0.001$) placed in their cages (**Fig. 2A**). More marked was the complete absence of mounting attempts *B3gnt1* null males made ($p<0.001$) within any of the trials (**Fig. 2B**). Any sniffing initiated by the *B3gnt1* null males towards the females was not sexual in nature at all. It was more of a general interest in the 'intruder' and some grooming were observed. None of the aggressive, anogenital, sexually specific interest displayed by wildtype controls was seen with the *B3gnt1* null males.

Since the sexual behavior of male mice relies on olfactory cues detected through the main olfactory system (Dulac 2000; Keverne 2004; Yoon et al. 2005), which is disorganized in the *B3gnt1* null mice (Henion et al. 2005), we have compared the mating behavior of *B3gnt1* null males to that of MOE-lesioned wildtype males via treatment with 2,6-dichlorobenzonitrile. This toxicant induces tissue-specific lesioning of the MOE, while leaving the VNO and their connections with the accessory olfactory bulb intact. The examination of anosmic wildtype males showed a similar mating behavior to that of *B3gnt1* null males, indicating that the reduced reproductive success of *B3gnt1* null males is likely related to axon disorganization that persists in the MOE of the null mice (**Fig. 2A, B**).

The reduced reproduction rate together with a lower Mendelian distribution of the null allele pointed to the possibility of a combined behavioral and physiological fertility phenotype. Additionally, intercrosses between male and female mice heterozygous for the *B3gnt1* null allele yielded fewer homozygous null animals than the 25% Mendelian distribution predicts (**Table 2**). The same bias against the null allele was observed when breeding *B3gnt1* heterozygous mice on the inbred C57Black/6 and outbred NMRI background ($p<0.001$). Although the *B3gnt1* null mice born from such matings were apparently normal, the shift in Mendelian inheritance could be related to an additional fertility defect. Genotyping at embryonic days

E3.5 and E10 showed that the distribution of the null allele was Mendelian at E3.5 while the bias observed in the pups detectable by E10 (**Table 2**).

The testes of *B3gnt1* null males were morphologically similar in weight and size to those from their wildtype littermates (data not shown). Histological examination of testis sections revealed no structural abnormality in the *B3gnt1* null male testes and all stages of spermatogenesis were discernable (**Fig. 3A**). The amount of mature sperm available for mating was quantified by releasing caudal epididymal sperm and counting with a hemocytometer. The average number of sperm collected from wildtype (n= 23) vs. *B3gnt1* null (n= 11) males was 1.7 and 1.1 x 10^7 per epididymis, respectively. Furthermore, *B3gnt1* null sperm were morphologically identical to wildtype sperm and motility was not impaired (data not shown). Testosterone production was also normal in *B3gnt1* null males as similar serum testosterone levels were measured in *B3gnt1* null and wildtype males (data not shown).

Certain sperm proteins known to play a role in egg binding activity carry N-glycosylation sites (Stein et al. 2005; Zhang and Martin-DeLeon 2001). To estimate whether the glycan chains of these proteins were affected by the disruption of the *B3gnt1* gene, we analyzed the ADAM (A Disintegrin and Metalloproteinase) proteins 2 and 3 and the hyaluronidase protein PH-20 (**Fig. 3B**) by Western blotting. We found that none of these proteins were altered in their molecular weight or in their expression level in *B3gnt1* null sperm, thus ruling out an involvement of these glycoproteins in the phenotype of *B3gnt1* null mice. We did not find any difference in the time or efficiency of the acrosome reaction when wildtype and *B3gnt1* null sperm were tested with or without the addition of the calcium ionophore A23187 (**Fig. 3C**). In vitro assays showed no difference in *B3gnt1* null sperm's ability and time needed to pass through the cumulus layer surrounding egg clutches and similar numbers of sperm were found attached to the eggs' zona pellucida (data not shown). Next,

we quantified sperm after entering the uterus. When *B3gnt1* null males plugged wildtype females, a normal number of sperm were ejaculated into the uterus although successful procreation was lower ($p<0.05$) (**Table 3**).

Embryo development from fertilization to the 2-cell stage was observed *in vitro* to assess the quality of the maternal oocytes and possible imprinting effects from the time of egg penetration to the 2-cell stage of embryogenesis. There was no significant difference or latency in the development of 2-cell stage embryos sired by wildtype vs. *B3gnt1* null males when mated with superovulated wildtype female (**Fig. 4**). Most of the embryos had reached the 2-cell stage at 22.5 h post coitus. Similarly, the number and morphology of blastocyst-stage embryos was identical when collected at E3.5 from superovulated wildtype females plugged by either wildtype or *B3gnt1* null fathers.

DISCUSSION

Our investigations showed that the reduced rate of reproduction observed in *B3gnt1* null males could not be attributed to any physical defect in their reproductive organs. Examination showed that spermatogenesis was intact and viable sperm was available. Our initial observations revealed a lower rate of reproduction in *B3gnt1* null males and a reduced rate of transfer for the *B3gnt1* null allele. There was a distinct possibility that these two events were linked. However, there was no link to the sex of the null allele contributing parent, as heterozygous animals mated with wildtype conspecifics of both genders showed the same shift. Hence no connection was found between the reproductive capacities of males as a result of imprinting of the *B3gnt1* gene during early embryonic development. We were able to show that the loss of homozygous null animals occurs between E3.5 and E10, possibly due to failed implantation since no embryo resorption sites could be detected at later stages of development.

Previous studies with the *B3gnt1* null mouse revealed defects in the pathfinding of olfactory neurons and of migrating GnRH-neurons (Bless et al. 2006; Henion et al. 2005). The areas affected, namely the olfactory and the GnRH-neurons of the hypothalamus, are essential for detecting and processing signals involved in regulating sexual behavior and spermatogenesis, respectively. Our study suggests that the reduced reproductive rate of *B3gnt1* null males is due to a failure to interpret and react to the olfactory cues emitted by ovulating females.

In mice, sexual behavior is highly influenced by odors detected as pheromones or other scents. Classically, the MOE has been deemed the area of the olfactory responsible for detecting common conscious scents while the processing of behavior influencing scents, like pheromones, was attributed to the VNO. Recently,

researchers showed that the MOE is essential to processes and signaling generally attributed to the VNO (Dulac 2000). Additionally, Yoon et al were able to show that the MOE modulates signaling cascades influencing GnRH-neurons in the hypothalamus. These neurons have been implicated in sexual behavior repeatedly (Boehm et al. 2005; Rajendren 2001; Wray 2002).

The role of the MOE and VNO in regulating male sexual behavior has been an area of debate for some time. Male mice lacking genes only found in the VNO, such as *Trp2* (Leypold et al. 2002; Stowers et al. 2002) showed no defects in initiating sexual contact to females. However, these males displayed sexual behavior towards male mice too. This suggests, as Keverne points out (Keverne 2002), that the VNO is not essential for the sexual behavior as such, but plays a role in sex discrimination. This leaves us with the possibility that at least some sexual behavior cues are under the control of some other part of the brain, independently of VNO function.

Gene-disrupted mice showing impaired sexual behaviors consecutive to deficits in the MOE have been described previously. Examples include the *Cnga2* (Mandiyan et al. 2005) and *Ac3* (Wang et al. 2006) null male mice. *Cnga2* is a cyclic nucleotide-gated channel necessary for odor-induced MOE signaling, while *Ac3* is a type 3 adenylyl cyclase coupled to odorant receptors via a G-protein olfactory subunit found in the MOE, and is not expressed in the VNO. Both *Cnga2* and *Ac3* null males have deficiencies in the amount of time they spend investigating females and show virtually no attempts to mount female conspecifics. The VNO in these animals is not affected by the gene disruption, but their mating behavior is. Notably, the *Cnga2* null males were not inclined to interact sexually with males either. Both the *Cnga2* and *Ac3* null models, along with the *B3gnt1* gene disruption, point to faulty MOE signaling for loss of sexual behavior.

As far as the reproductive machinery was concerned, there was no reason for the *B3gnt1* null males not to procreate. It is likely that some of the null males were able to learn the behavior required to copulate with females by observing other males, which may account for the low rate of reproduction observed. However, this does not mean that those *B3gnt1* null males can discern ovulating females. Our comparison of naive males, with and without MOE lesions, show that there is a striking difference in their response to estrous females. Further studies into the processes at work here are topics of ongoing investigations.

Although no hormonal or gonad development defects were observed in the *B3gnt1* null males, the delicate balance between olfactory development and normal reproductive capacity is apparent. There are gene products that decrease male sexual behavior without any direct link to the MOE or migrating GnRH-neurons. Many complex signaling mechanisms involving hormonal and neuronal signaling potentially modulate murine male sexual behavior. Activin type-II null males displayed a similar impairment in their sexual response to female mice (Ma et al. 2005). However, unlike the *B3gnt1* null males, these mice had multiple physiological disorders in the male reproductive organs in addition to the fact that they mated less frequently than wildtype males. *B3gnt1* offers a new target in this list of possible causes of the olfactory-linked behavioral or anosmic phenotypes described. Like *Cng2* and *Ac3*, *B3gnt1* represents a gene expressed in the MOE. Other proteins with functions in GnRH-neuron guidance or expression in the developing olfactory continue to give us clues as to how these two intimately linked developmental processes fit together.

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FIGURE LEGENDS

Figure 1. Loss of poly-N-acetyllactosamine chains on thymocytes of 8-12 week old *B3gnt1* null mice. Thymocytes from wildtype and *B3gnt1* null were stained with FITC-conjugated *Lycopersicon esculentum agglutinin* (LEA), concanavalin-A (ConA) and Jack bean lectin (Jac). *B3gnt1* null thymocytes displayed a loss of FITC-LEA staining due to lack of poly-N-acetyllactosamine structures. FITC-ConA and -Jacalin stained thymocytes obtained from wildtype and *B3gnt1* null animals with equal intensity.

Figure 2. Aberrant sexual behavior of *B3gnt1* null males. Two separate tests were scored according to protocols described in *Materials and Methods* to assess how much time males spent investigating (sniffing) and how often they attempted to mount their conspecifics (wildtype[WT], n=4; *B3gnt1* null [KO], n=4) . Both tests revealed a deficient sexual response by the *B3gnt1* null males to estrous females. This lack of male sexual behavior was comparable to behavior demonstrated by MOE-lesioned wildtype male mice when chemically ablated (wildtype, n=3; MOE-lesioned, n=5)

Figure 3. In vitro analyses of wildtype vs. *B3gnt1* null testes. A) Hematoxylin and eosin staining of 3 um paraffin sections of wildtype (WT) and *B3gnt1* null (KO) testes were morphologically comparable. All stages of spermatogenesis are discernable. B) Western blotting analysis of ADAM-2, -3 and PH-20 expression in sperm lysates prepared from age matched wildtype and *B3gnt1* null males. C) Acrosome reaction of caudal epididymal sperm from wildtype and *B3gnt1* null males scored by staining with Coomassie Blue. Reaction times were compared in the presence (+) or absence (-) of the ionophore A23187. The bars show the average values + SEM.

Figure 4. Two-cell embryo development in eggs fertilized by *B3gnt1* null males. Wildtype and *B3gnt1* null males were mated with estrous females and checked for vaginal plugs. Fertilized eggs were removed from plugged females the same evening, and the embryos cultured accordingly. The development of these embryos was followed under the microscope in hourly intervals and the number of embryos progressing was noted. The time course shows that the 2-cell stage of development was reached by most wildtype (solid line) and *B3gnt1* null (dotted line) sired embryos by 22.5 h post coitus (wildtype, n= 49; *B3gnt1* null sired, n=62 developing zygotes).

Table 1. Number of litters sired per month

Months[a]	1	2	3	4	5	Expected	Observed	Litter size
Wildtype (n=4)	14	15	13	15	14	80	71 (88.8%)	7.5 ±2.5
B3gnt1-null (n=15)	1	4	3	5	1	300	14 (4.67%)	8.4 ±2.4

[a] Each male was housed with a wildtype female for a week, giving up to four mating opportunities per month.

Table 2. Inheritance of the *B3gnt1* null allele from *B3gnt1* heterozygous matings.

Genotype expected frequency (%)	+/+ (25%)	+/- (50%)	-/- (25%)	
Newborn (NMRI)	35%	48%	17%	(n=268)
Newborn (C57Bl/6)	33%	51%	16%	(n=913)
E10 (C57Bl/6)	32%	57%	11%	(n=53)
E3.5 (C57Bl/6)	26%	52%	22%	(n=151)

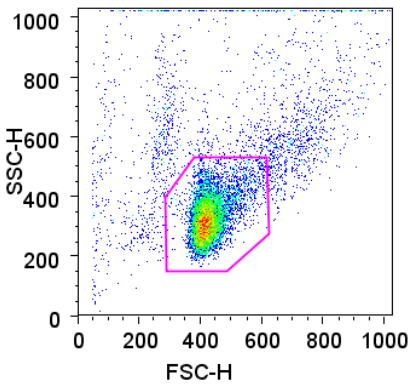
Table 3. Ejaculated sperm count and number of litters obtained from mated females.

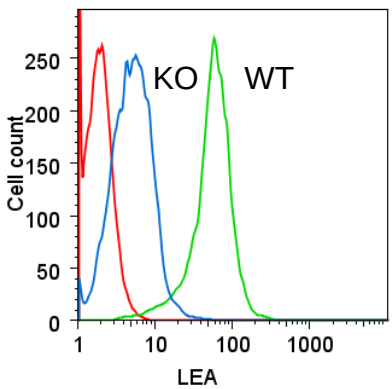
Genotype	Ejaculated sperm count (x 10^6)	No. of litters from mated ♀‡
wildtype	1.14 (n=3)*	19 / 22
B3gnt1 null	1.30 (n=10)*	18 / 31

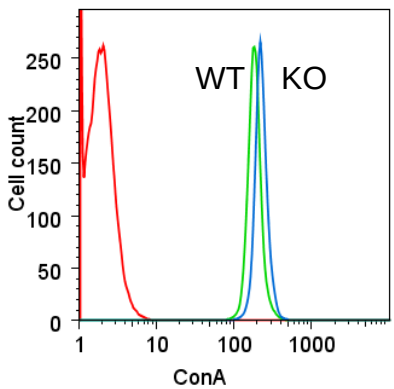
*n refers to the number of males tested.

‡ $p<0.05$

Figure 1 – Biellmann *et al.*







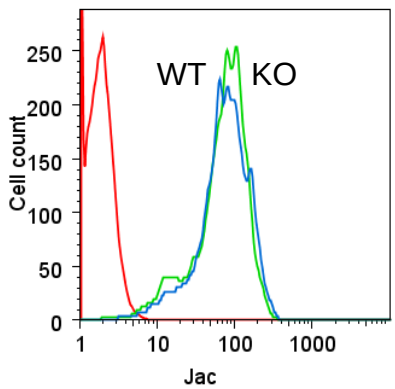


Figure 2 – Biellmann *et al.*

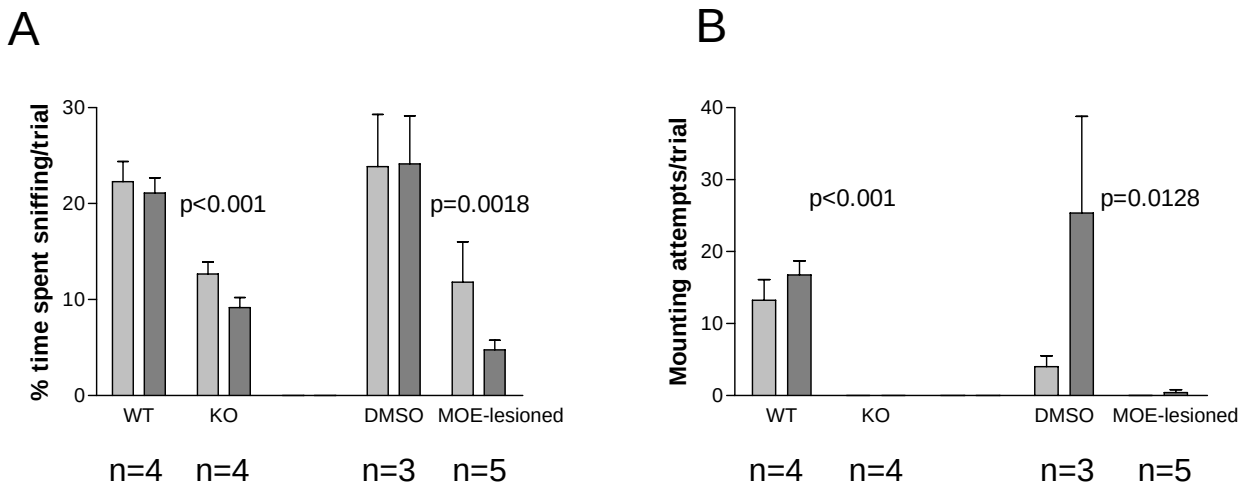


Figure 3 – Biellmann *et al.*

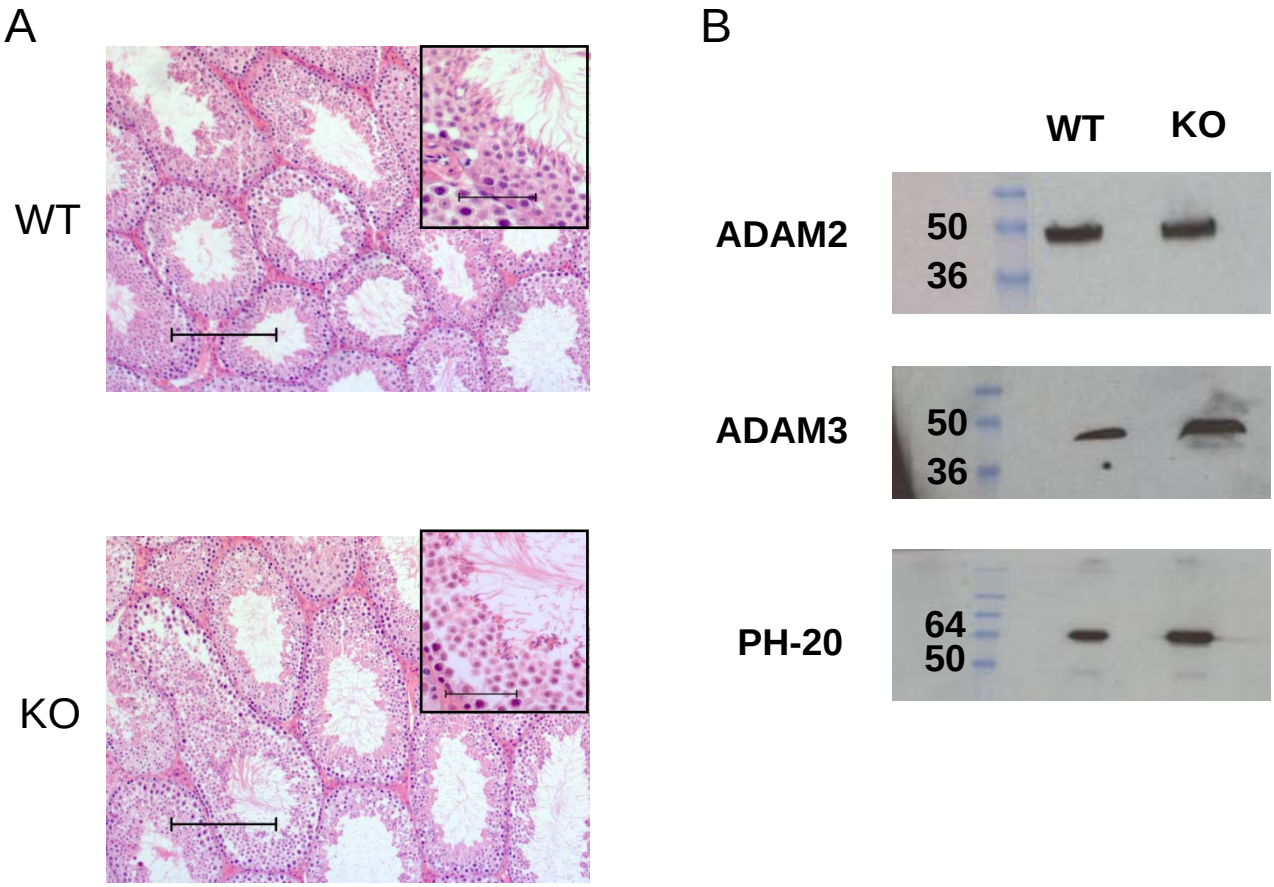
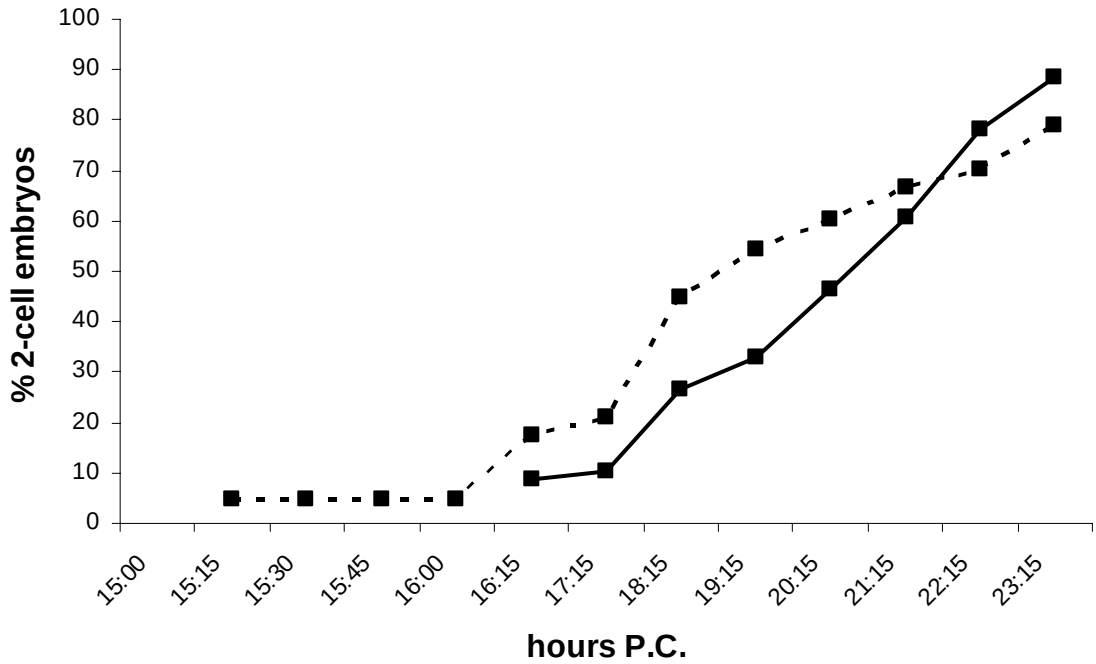


Figure 4 – Biellmann *et al.*



Manuscript 2:

Results obtained thus far for the 3GNT5 null mouse created as part of this dissertation project. Submission will follow when glycolipid analyses have been completed.

Early embryonic expression of Lc3- synthase gene *B3gnt5* is essential to pre-implantation development of the murine embryo

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Abstract

Glycolipids are found throughout the plasma membranes of mammalian cells. Due to their ubiquitous expression, glycolipids have been linked to various cellular activities including trafficking, signal transduction, and inter/intracellular interactions. Glycolipid biosynthesis is a cascade controlled by highly specific glycosyltransferases. One of the key enzymes implicated in the initiation of lactoseries glycolipid biosynthesis is the β1,3-N-acetylglucosaminyltransferase V (β3GNT5) which is coded for by the *B3gnt5* gene in the mouse. In an effort to study the targets and effects of missing lactoseries glycolipids *in vivo* a heterozygous ES cell line was created to generate a *B3gnt5* null mouse line. The homozygous targeted deletion of *B3gnt5* led to the embryonic death of these mice. The data collected concerning the survival of null mice suggests that the expression of *B3gnt5* is crucial to the embryonic development of the mouse embryo. Additionally, our findings show that *B3gnt5* transcript is detectable throughout the pre-implantation stage of murine development. Finally, we investigated the expression of the gene in wildtype and heterozygously targeted ES cells to gain insight into the role of this gene on murine ES cell multipotency which might be relevant to the pre-implantation lethality observed.

Introduction

The β1,3 *N*-acetylglucosamine transferase V (β3GNT5) has been described as a lactosylceramide modifying enzyme responsible for the elongation of lacto-series mammalian glycolipids leading to the synthesis of the Lc3 structure [1, 2]. Glycolipids are generally divided into two classes according to their main structural cores. Both of these main cores are ubiquitous. Some glycolipids, including those modified by β3GNT5, are built on glucosylceramide (Glc-Cer) while others have galactosylceramide (Gal-Cer) as a scaffold for further glycan modification. After the addition of a galactose residue to the Glc-Cer scaffold, lactosylceramide (Lac-Cer) results, a precursor for hundreds of glycolipids. Glycolipids synthesized on the Lac-Cer precursor are subdivided into three major classes, the ganglio-series, the globo-series, and the lacto-series (fig. 1). The glycosyltransferase controlling the formation of the Lc3 structure represents the first modification selecting these glycolipids as lacto-series glycolipids.

Glycolipids have only gained attention in the mid twentieth century [3]. Glycolipid research was limited due to lack of techniques available to characterize these structures at all, let alone their functions. As a result, elucidation of glycolipid structures *per se* and their expression profiles *in vivo* were the focus of many investigations, with their functions initially taking a back seat. Since then, glycolipids have been implicated in processes ranging from embryonic and neuronal development to cellular adhesion and as viral receptors mediating infection [4, 5]. One of the first properties attributed to the glycolipids was their species patterns on erythrocytes [6]. The discovery of the developmentally- and differentiation-specific expression of glycolipids quickly followed [7-9]. Here it was shown that varying glycolipid structures are expressed in the murine embryo from the embryo pre-implantation stages. There are several glycoepitopes and glycolipids which have been implicated in successful embryonic development [8, 10-16].

Collectively these are termed the stage specific embryonic antigens (SSEA) and have been shown to undergo rapid changes in their epitope expression [3]. SSEA-1 (also called Lewis X) is perhaps one of the most well characterized members of these antigens. The glycolipid core that gives rise to the SSEA-1 depends on the lacto-series core for its biosynthesis on glycolipids, which is previously modulated by β3GNT5. However, the SSEA-1 is a carbohydrate epitope that can be found on glycolipids, glycoproteins, and proteoglycans. Its function is believed to be the regulation of cell adhesion in embryogenesis, cell differentiation, and development of the neuronal system [7, 17].

The availability of murine embryonic stem (ES) cell technology had provided a way to investigate the functional role of the glycosyltransferases involved in glycolipid biosynthesis *in vivo*. Studies on mice missing genes encoding glycolipid-modifying glycosyltransferases have displayed a wide variety of phenotypes [5, 18]. Deletion of the complex ganglioside (GM2/GD2) synthase gene in mice led to axonal degeneration in optic and sciatic nerves and decreased central myelination. This underlines the importance of glycolipids as mediators of cellular adhesion. When the more basic cores of glycolipids were examined in null mice models, the phenotypes were surprisingly variable. While deleting the galactosylceramide transferase gene (*Cgt*) had little effect on embryonic development, it led to numerous adult phenotypes due to the role of sulfatides in myelin function and stability. These included tremors, mild ataxia, and progressive hind limb paralysis [19]. Another group disrupted the glucosylceramide gene (*Ugcg*). Homozygous null embryos died by embryonic day (E) 9.5. Moreover, their lagging development was visible earlier [20].

The crucial role of glycolipids in early embryonic development was also shown in a β1,3 N-acetyl-galactosamine- transferase (B3GalNacT) null mouse which lacked Gb4. This

B3GalNAcT was later shown to be the enzyme coded by the *B3galnt1* gene (Accession number NM_020026) controlling Gb4 synthesis [21], and the absence of this class of glycolipid led to pre-implantation death of the murine embryos [22]. These studies show the broad effect the glycolipids have on murine phenotypes, and their particular importance in early embryonic development. While the disruption of an entire class of glycolipids was certainly expected to cause severe phenotypes, the studies show that the time and specificity of the defects caused by missing glycolipids varies. The importance of functional glycolipids in early embryonic development and in adult mice has been shown for both core glycolipid precursors and their downstream products.

Among the functions attributed to the *B3gnt5* gene encoding the Lc3 synthase (β3GNT5) was when *Chou et al.* showed that it was an enzyme key to the regulation of sulfoglucuronylglycolipds (SGGL) in the developing nervous system [23]. The SGGL epitopes are expressed in a highly regulatory fashion during rat embryonic brain development and again postnatally [24-27]. In addition SGGLs are reactive to the monoclonal antibody HNK-1 which recognizes terminal nonreducing carbohydrate 3-sulfoglucuronic acids of the glycolipids. In adult rats, *B3gnt5* expression is limited to the Purkinje cells in the cerebellum. The developmentally regulated expression of the SGGLs coincides with certain cell migration and differentiation phases [28]. The effect of the SGGLs on neuronal development makes them prime targets for research in many areas including brain plasticity and neuronal deficits. At the same time SGGLs provide us a new target for examining the role of glycolipid function *in vivo*.

Further investigations into the expression of *B3gnt5* revealed that the gene is highly expressed in spleen and placenta as well as in various organs in the developing murine embryo after embryonic day 11 (E11) [1]. In order to study the physiological significance

of the lacto-series glycolipids initiated by *B3gnt5,* we have generated a mouse missing the *B3gnt5* gene. Since the Lc3 substrate is the scaffold for lacto-series glycolipid elongation by different glycosyltransferases, the absence of this structure could provide us insights into its structural and functional properties. As this gene is present already in the embryo, its role in early murine development and differentiation was the main focus of our investigations. Our investigations suggest an essential role for *B3gnt5* in early embryonic development.

Materials and Methods

Gene targeting:

Mouse Genomic 129/Sv bacteriophage library (Stratagene), was screened with a mouse *B3gnt5* cDNA probe. A genomic clone of 12 kilo base pair (kbp) was isolated and subcloned as a NotI-NotI fragment in pBluescript II. A 3.6 kbp long arm fragment from the coding exon for *B3gnt5* was cut from the genomic sequence containing plasmid with a BstX I/ Xba I digest and cloned into the neomycin resistance- (*neo*^{r}) containing vector (PGKneoBPA) at the EcoR V site in front of the *neo*^{r} cassette. The short arm of the *B3gnt5* coding exon was cloned into pBluescript II SK+ as a 1.7 kbp BstX I/ Nde I fragment at the Sma I site of the polylinker. The 1.7 kbp fragment containing pBluescript II SK+ plasmid was recloned into pBluescript II SK+ as a Sac II- Xho I fragment to obtained desired restriction sites for further modification The orientation of the fragment was verified with sequencing, showing that the short arm fragment was now flanked by 2 Not I restriction sites. A Not I digest was done, and the 1.7 kbp fragment ligated into the Not I site following the poly A site for the *neo*^{r} cassette of the long arm containing PGKneoBPA vector. As a result we created a targeting vector where the *neo*^{r} sequence replaces a 745 bp stretch of the *B3gnt5* coding region (**Fig. 1A**). Restriction digests were done to insure proper orientation of the fragment and sequencing of the genomic region flanking the *neo*^{r} gene were done to insure proper genomic sequence was in the targeting vector. The final targeting vector was 7.1 kbp in size. 10 μg of the vector was linearized using Sac II, and transformed into five million TC1 and R1 murine 129/SvEv embryonic stem (ES) cells.

Disruption of *B3gnt5* in ES cells and generating *B3gnt5* chimeric mice:

ES cells containing the transformed *neo*^{r} construct were selected for using neomycin (G418) (200 μg/ml) supplemented KO DMEM (Gibco) containing 15% fetal calf serum (Gibco), and 1000U/ml leukemia inhibitory factor (ESGRO). After several days of selective culture, 300 *neo*^{r} clones were re-cultured in 24-well plates using the same selective media. Primers were constructed so that the region flanking the end of the *neo*^{r} insertion and the genomic sequence were detectable as a 1920 bp fragment in the null allele of homologously recombined ES clones. The primers had the following sequence, (5' - TAC TAC CCT GTC TAG GAG CAG TTG - 3' and 5'- CAT CGC ATT GTC TGA GTA GGT GTC - 3') and were run with an initial denaturing step at 94° C for 5 min followed by 35 cycles of 94° C 45 sec, 52° C 1 min, and 72° C 1 min 50 sec, with a final elongation at 72° C 5 min. The proper recombination of the *B3gnt5* allele was confirmed using genomic Southern blot for which a ^{32}P labeled 0.5 kbp sequence from B3gnt5 genomic sequence was used as a probe. Eco RI digested wildtype and possible heterozygous ES clones were probed. Finally the positive clones were karyotyped using the standard potassium chloride method [29]. Two chimeric males resulted from the injection of ES cells heterozygous for the *B3gnt5* allele into blastocysts derived from C57BL/6 mice. These males were then bred with C57BL/6 females and the resulting offspring were genotyped for the transmission of the null allele. Offspring resulting form both founder males were bred onto the C57BL/6 for 6 generations. Finally, F6 heterozygotes were crossed in order to obtain homozygous null mice.

PCR genotyping:

To detect the *B3gnt5* null allele in viable offspring, a 789 fragment containing a portion of the genomic and neo^r sequence was amplified (5' - ACT CGT CAA GAA GGC GAT AGA A - 3' and 5' - CGG CCA TTG AAC AAG ATG GAT T - 3'). For the wildtype

allele, primers amplifying a region disrupted by the *neo*^{r} insertion were used to amplify a 651 bp fragment (5' - GGC TCA AGA TGT CCT CCT CTT A - 3' and 5' - ACA TGG TCC TGT GGC AAG ATT C - 3'). These PCRs were both run with an initial denaturing step at 94° C for 5 min, followed by 35 cycles of 94° C 1 min, 60.5° C 45 sec, 72° C for 40 sec, and then a final extension at 72° C for 5 min. To genotype E3.5 blastocysts, (if E0.5 is the time of plug detection), primers amplifying a smaller fragment of the null allele were used. Here, a 326 bp fragment of the *neo*^{r} cassette was amplified for the *B3gnt5* null allele (5' - CAT CAG CCG CTA CAG TCA AC-3' and 5'-CAT CAG AGC AGC CGA TTG TC - 3'), while the same primers used to detect the wildtype allele as described above (651 bp) were used. REDTaq^{TM} (Sigma) genomic DNA polymerase was used to amplify DNA from blastocysts. The PCRs were run with an initial denaturing step at 94° C for 5 min, followed by 35 cycles of 94° C 45 sec, 63.5° C 40 sec, 72° C 25 sec, and a final extension at 72° C for 5 min.

Isolation of E3.5 and E10 Embryos:

Pre-implantation (E3.5) and E10 embryos were collected from pregnant females. E3.5 blastocysts were isolated by flushing of the uterine horn. Blastocysts were placed in gelatinized 96-well plates for 5-7 days. This allowed for the outgrowth of enough cells for proteinase K treatment (25 ug/ml) and direct genotyping with the primers described above. E10 embryos were collected individually from their placentas being careful to remove all maternal tissue. E10 embryos were placed in proteinase K solution immediately, digested O/N at 56°C and the DNA phenol chloroform extracted before being subject to PCR genotyping.

In Situ Hybridization:

Pre-implantation embryos collected for *in situ* hybridization were obtained from wildtype C57BL/6 matings with superovulated females. Superovulation was carried out as previously described [30]. Embryos were removed at E1.5 (2-4-cell stage) and E3.5 (blastocyst stage). To obtain embryos at the 4-cell up to the morula stage, 2-cell embryos were cultivated in M16 media (Sigma) until the desired stage was reached. A protocol describing *in situ* hybridization conditions for whole murine embryos [31] was modified for our experiments as follows. All solutions were made with RNase free reagents and with DEPC-treated water. Embryos were fixed for one hour at RT in 4% PFA and washed twice in 0.1% Tween PBS (PBT). Embryos were dehydrated in increasing concentrations of methanol in PBT (25%, 50%, 100%) for 5 minutes each and stored in 100% methanol until use at -20° C.

Before use the embryos were rehydrated in the opposite methanol concentration order (100%, 75%, 25%) and then washed twice in PBT. For permeabilization the embryos were incubated in Radioimmunoprecipitation (RIPA) buffer (Sigma) buffer for 10 min and then rinsed in three changes of PBT for 5 min each. Embryos were then washed in a 1:1 mixture of PBT/hybridization solution (supplementary data) for 10 min and then again in just hybridization solution. Prehybridization in hybridization solution followed at 70° C for ≥2 h. The embryos were hybridized with either sense or antisense riboprobe over night in 750 ul hybridization solution at 70° C.

Embryos were washed in hybridization solution followed by 3 washes in solution I (supplementary data) all at 70° C except the last which was at 65° C. Three 30 min washes in solution II (supplementary data) at 65° C followed. Embryos were cooled to room temperature and subsequently washed 3 times in maleinic acid buffer (MAB)

(supplementary data) buffer. A blocking was done with 2% Boehringer's Blocking Reagent (Roche, Switzerland) in MAB for 90 min at RT. Finally pre-absorbed anti-DIG-AP antibody was added to the embryos and incubated over night at 4° C. The next day embryos were washed three times 5 minutes at room temperature with MAB followed by five 60 minute washes with MAB at room temperature. Embryos were incubated with 1x levamisole MAB over night at 4°C.

Embryos were washed three times 10 min in fresh AP (supplementary data) buffer at RT. Staining was done at room temperature or 4°C for longer incubations (O/N). When color development was sufficient, i.e. when antisense probe gave visible signals under the microscope, the reaction was stopped with 2 mM EDTA in PBT. On the last day, embryos were post fixed in 4% PFA/ 0.1% gluteraldehyde in PBT for 1 h at RT or over night at 4°C and then washed twice in PBT. Embryos were washed in glycerol: PBT (1:1) and stored in glycerol: 2 mM EDTA in PBT (4:1). The signals observed at various stages of development were documented using the Zeiss Axiovet 200M microscope (Carl Zeiss AG, Feldbach, Switzerland).

For probe preparation, plasmid containing the cDNA sequence needed for the production of in situ riboprobes were available in our laboratory from previous experiments [1]. T7 and SP6 riboprobes were made using a DIG RNA labeling kit (Roche, Switzerland). Since both sense and antisense probes are on the same plasmid and differ only in their digests (Hind III for anti-sense and Sac I for sense) both riboprobes were 908 bp long. An alkaline hydrolysis followed the labeling reactions in order to reduce the size of the riboprobes to about 300 bp for better passage through the permeabilized embryos.

Quantitative PCR:

Quantitative PCR (qPCR) reactions were done to check for the expression levels of *B3gnt5* in wildtype versus heterozygous ES cell clones. Primers were designed for *B3gnt5* and an RNA polymerase gene (NP_033115) which was used as a reference marker. The following primers were used for the *B3gnt5* gene (5' - CAT ATA CCC ACA GAC CAG AG - 3' and 5' - CAC AGA GCT GTG CTT GAG AG - 3') and the RNA polymerase gene (5' - AGC CAA AGA CTC CTT CAC - 3' and 5' - GGG GTT AGG GTC ATA GTA GA -3'). As the protein coding region of *B3gnt5* is found in a single exon, RNA batches used for cDNA production were DNase treated and tested for the presence of DNA contamination with a standard PCR reaction. 2 μg DNA-free RNA was used to make cDNA using a reverse transcription kit Omniscript RT (Qiagen). 2 μl (10% of RT reaction) of the cDNA produced was used in the qPCR reactions with the SYBR® Green JumpStart kit (Sigma). The reactions were measured in MX-300P QPCR machine (Stratagene). The reactions were run with an initial denaturing step of 10 min at 95° C followed by 40 cycles of 95° C 15 sec, 59° C 1 min, and 72° C 20 sec. A final cycle of 95° C 1 min, 55° C 30 sec, and 95° C 30 sec was run after the 40 cycles described.

Results:

The targeting construct (**Fig. 2**) used in the electroporation of R1 and TC1 ES cells from 129/SvEv mice led to the production of four independent ES cell clones which were heterozygous at the *B3gnt5* gene locus. These independent ES cell clones were confirmed using both PCR and genomic Southern blot analysis. The genomic Southern probe distinguished between the wildtype (3 kbp) and the null allele insertion (2.3 kbp) (**Fig. 3A**) in these Eco RI digested ES clone's DNA. Finally, the ES cells used for blastocyst injection were karyotyped to show they contained a complete set of 40 chromosomes (data not shown).

Properly recombined ES cells were injected into the blastocysts of C57BL/6 mice to produce chimeric male offspring. Two chimeric founder males were obtained. These males were mated with wildtype C57BL/6 females and the F1 progeny screened with PCR for germ line transmission of the disrupted allele as described in materials and methods. Both chimeric males transmitted the null allele. Offspring positive for the disrupted gene were backcrossed onto the C57BL/6 background for 6 generations. All the heterozygous offspring obtained from the chimeric males were grossly normal. Both male and females carriers of the null allele were obtained. F6 heterozygotes were then bred to produce null offspring. After crossing 17 independent heterozygote breeding pairs, no homozygous null offspring were detected (**Table 1**). A previous investigation showed *B3gnt5* expression was detectable at numerous stages of embryonic development, especially at E11, and in the placenta [1]. Mid-gestation embryos (E10) were isolated from pregnant females from heterozygous matings. (**Fig. 3B**). Fifty one embryos from seven heterozygous matings revealed no homozygous null embryos at E10 (**Table 1**). Notably, no resorbed embryos were observed during the E10 isolations. In an effort to identify the time point at which *B3gnt5* causes lethality we examined embryos prior to

uterine implantation (E3.5) at the blastocyst stage. Again, no homozygous null embryos were detected (**Table 1**) with PCR (**Fig. 3B**). Our results suggest *B3gnt5* expression is important for very early embryonic development in the mouse.

Detection of the *B3gnt5* transcript in various early development sages with RNA *in situ* hybridization followed. For clarity, we tested embryos of the wildtype C57BL/6 background on which our null allele was introduced. Our investigation revealed that mRNA transcript of the gene was expressed as early as the 2-cell stage (**Fig. 4**). In fact all the wildtype embryos were positive for *B3gnt5* at all stages tested up to the blastocyst. These findings suggest *B3gnt5* is expressed during the pre-implantation stages of embryonic development might be important for early embryonic development in the mouse.

Finally, since we could see a very pronounced staining of the inner cell mass of the blastocyst with *in situ* hybridization, we examined our heterozygous ES cells for altered *B3gnt5* gene expression. Quantitative PCR studies were performed on cDNA reverse transcribed from the mRNA obtained from wildtype TC1 and R1 ES cells and their respective heterozygous clones. A decrease in the level of mRNA expression in the heterozygote ES cells points to a possible role for *B3gnt5* in multipotency and consequently might affect early embryonic development. According to our data *B3gnt5* mRNA was detectable in wildtype ES cells, although not at an elevated level when compared to the house keeping gene (RNA polymerase). Our findings revealed a much lower level of *B3gnt5* mRNA expression in the heterozygote ES cells when compared to their wildtype counter parts. For the R1 line, the heterozygous ES cell line showed only 14.8% (± 3.95%, n=3) of the normal wildtype *B3gnt5* mRNA expression levels. In the heterozygous line derived from the TC1 ES line, the mRNA expression level was at only

roughly 3% (± 0.21%, n=3) of what was observed in the wildtype line. The disruption of just one *B3gnt5* allele is enough to decrease mRNA substantially. The effect of these reduced transcript levels on lactoseries glycolipid biosynthesis is currently under investigation.

Discussion

Glycolipids are poorly understood biological molecules which can be found throughout mammalian membranes. By disrupting the *B3gnt5* gene we aimed to get a better understanding of the functions of the lacto-series glycolipids *in vivo*. The mutation of this gene proved to be fatal for the mice, and the time point at which it causes embryonic death and its possible role in multipotancy was examined. Our investigations of the developing embryo first showed that null embryos were not detectable at E10, the day before significant levels of *B3gnt5* had been shown in previous Northern blot experiments and whole mount in situ experiments [1]. This suggests the expression of *B3gnt5* is crucial to earlier stages in murine embryonic development. Here we report that mice which do not express *B3gnt5* die prior to the pre-implantation (E3.5) stage of development. Consequently, the regulation and expression of *B3gnt5* is likely to be essential for processes prior to cell division or at the very latest for compaction at the morula stage.

We were able to detect *B3gnt5* mRNA transcripts as early as the 2-cell embryonic stage in wildtype murine development. Based on current information concerning the precursor necessary for β3GNT5 activity, namely glucosylceramide transferase (*Ugcg*), the early embryonic phenotype observed came unexpectedly. Disruption of the *B3gnt5* gene led to a lack of blastocyst stage embryos although the *Ugcg* null embryos survived to E7.5-8.5. However, this is not the first example of such a discrepancy in the phenotypes observed in mice missing glycosyltransferases that are implicated in glycolipid synthesis. In their investigation of the effects of removing the Gb4-synthase gene (*B3GalNacT*), *Vollrath et al.* showed that disruption of this gene displayed a similar phenotype to the *B3gnt5* null mutation [22]. This leads us to question the availability of alternative glycolipid, or glycolipid-like structures upon which *B3gnt5* and *B3glaNAcT* encoded proteins are active

in the *Ugcg* null mice (**Fig. 5A**). Alternatively, it is likely that there is another Glc-Cer synthase which is active enough to afford a longer survival time in the *Ugcg* null embryos. Here it should be mentioned that there are two genes with considerable homology to *Ugcg* whose activity has not yet been described in the human genome [32, 33]. Although these homologs have not been reported yet in mice, they could also exist.

Another factor influencing the development of the developing embryo is the expression of maternal and/or paternal mRNA and proteins. It has been shown that certain maternal mRNAs can persist through to the blastocysts stage of development in the mouse [34, 35]. Since we could not detect a gap in the expression of *B3gnt5* from the 2-cell through the blastocyst stage of development, we cannot account for possible maternal RNA expression of the gene. The temporal inconsistencies displayed by embryonic lethality phenotypes in the glycolipid synthesis cascade may also be influenced by maternal or paternal expression of the genes in question. Moreover, the lack of blastocyst stage embryos we observed might be the result of a problem earlier in the fertilization cascade, for example, if the fusion of two null gametes is not possible at all.

Although there is no clear upregulation of the *B3gnt5* gene detectable with qPCR in murine ES cells, a constant level of expression is detectable. Moreover, a considerable drop in transcript in the heterozygous ES cell lines derived from both the TC1 and R1 lines was detectable. Although the qPCRs do not prove *B3gnt5* is important to early embryonic development, it does give us a quantitative indication of the severity of the heterozygous null phenotype. Apparently low levels of *B3gnt5* are enough to 'rescue' the animal in the heterozygous background even if these are at less than 15% of wildtype. Other glycans whose expression has been implicated in murine embryo development are the SSEAs. The difficulty in predicting the effects of altering embryonic epitopes in vivo

was shown in a recent study with the α1,3-fucosyltransferase IX null mouse (FUT9). Fut9 regulates the fucosylation of the SSEA-1 and was believed to the essential recognition glycan for the proper function of the SSEA-1 epitope. However, disruption of the FUT9 coding gene showed that although the null mouse completely lack the SSEA-1 structures during early embryonic stages, these mice develop normally [17]. Interestingly, the LC3 precursor controlled by β3GNT5 is a glycolipid precursor for this fucosylated epitope (**Fig. 5B**). It is possible that the recognition epitope formerly attributed to the entire SSEA-1 epitope is limited to Lc3, and hence relies on a functional *B3gnt5*.

Finally, the discrepancy in the time of lethality between the *Ugcg* null and the mice missing down stream enzymes like *B3gnt5* is not clear. Clearly the existence of homologous genes which can code for compensating or competing enzymes has to continue. Additional investigations must also be done to clarify the issue of alternative substrates which might be modified by β3GNT5. If this gene is expressed continuously in the pre-implantation embryo, its source must be clarified. The question of whether a maternal transcript is involved remains. In addition β3GNT5 may be active in cascades other than Lc3 synthesis. However, even if Lac-Cer is its only acceptor substrate, any products relying on this glycolipid structure will suffer from its loss. Neither the absence of lactoseries glycolipids nor the accumulation of ceramide metabolites has been shown to lead to the lethal phenotypes observed. Before a the molecular mechanism causing the embryonic deaths of these mice can be described and documented, a connection between the activity of these genes, their products, and the observed phenotypes has to be established.

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Figure Legends

Figure 1: Glycolipid biosynthesis based on the Gal-Cer and Glc-Cer core substrates

Glycolipids with the Lac-Cer core structure can be further divided after the modification of the Lac substrate into globo-series, lacto-series, and ganglio-series glycolipids. Notice that an alternative modification of the globo-series precursor is possible to give rise to the Gb4 structure. The *B3gnt5* gene investigated in this study codes for the β3GNT5 glycosyltransferase which catalyses the first step in lacto-series biosynthesis by adding a β1,3-GlcNac residue to the Gal terminal glycan of the Lac-Cer substrate.

Figure 2: Schematic depiction of the wildtype *B3gnt5* coding exon and the design of the targeting vector used to disrupt *B3gnt5*

The targeting vector was obtained by cloning a *neo*^{r} sequence cassette into the coding exon of *B3gnt5*.

Figure 3: Genomic southern and PCR genotyping B3gnt5 and B3gnt5 null alleles

A. A probe cut from the genomic sequence of B3gnt5 was used to identify the wildtype vs. the null alleles. ES cells which screened positive for neo^r in selective media and via PCR for the homologous recombination of the null allele were digested with Eco RI and probed to reveal the single insertion site of the targeting vector and the appropriate change in size. The null allele revealed a signal at 2.6 kbp while the wildtype allele gives a signal at 3.0 kbp.

B. PCR was used to genotype viable offspring, E10 embryos, and E3.5 embryos. The primers used for amplifying the wildtype and null fragments of DNA obtained from viable pups and E10 embryos are shown in the top gel. The smaller fragments amplified using a separate set of primers from the DNA isolated from E3.5 embryos are shown in the bottom gel

Figure 4: RNA i*n situ* hybridization of *B3gnt5* expression in embryos at various pre-implantation stages.

Sense and antisense RNA probes were made with a DIG label as described. All stages of the murine embryo show expression of *B3gnt5* preceding implantation into the uterus. The pictures show embryos taken from C57Bl/6 wildtype matings. There is definite constant expression of B3gnt5 transcript at all stages preceding uterine implantation.

Figure 5: Summary of disrupting glycolipid modifying enzymes *in vivo*

A. The embryonic phenotypes observed in mice with gene disruptions that code for enzymes in the glycolipid biosynthetic pathway are variable. The cumulative effects we would assume to occur based on the cascade in which these enzymes functions leaves us with inconclusive results concerning the glycolipids in early embryonic development. (Cer: ceramide)

B. The SSEA-1 embryonic epitope is dependent on the Lc3 core synthesized by the glycosyltransferase coded for by B3gnt5. Interestingly; the loss of its terminal fucose does not lead to any abnormalities in vivo. This suggests that an underlying structure is essential to its function. Candidates include the Lc3 and Lc4 structures depicted.

Table 1: Genotype analysis of *B3gnt5* heterozygous crosses at various stages of murine development

Developmental Stage	WT (+/+)	Het (+/-)	KO (-/-)	Total screened
Blastocyst (E3.5)	20.8%	79.2%	0	48
Embryo (E10)	27.4%	72.5%	0	51
Viable pups	34%	66%	0	205

Figure 1 Biellmann *et. al.*

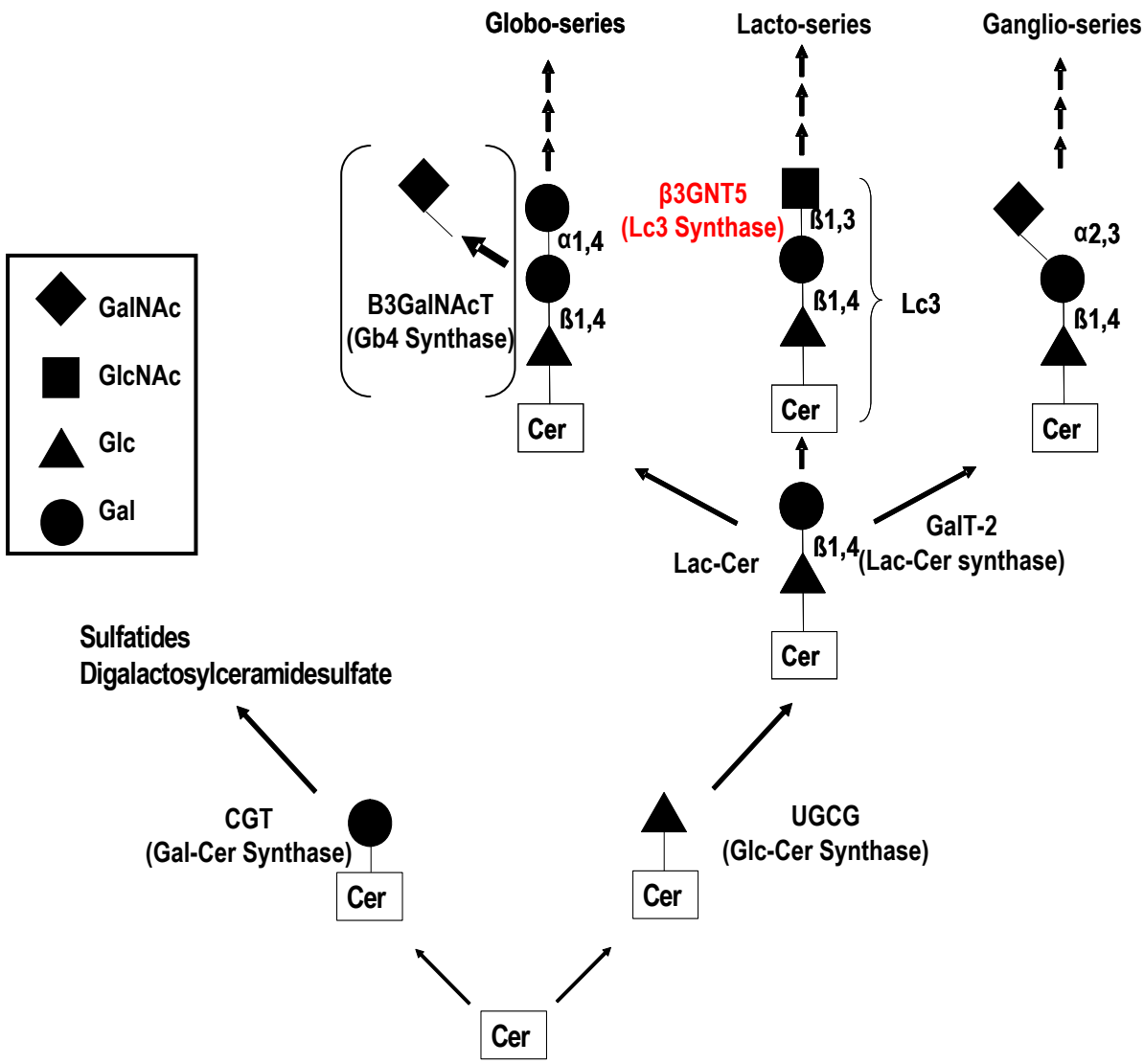


Figure 2 Biellmann *et. al.*

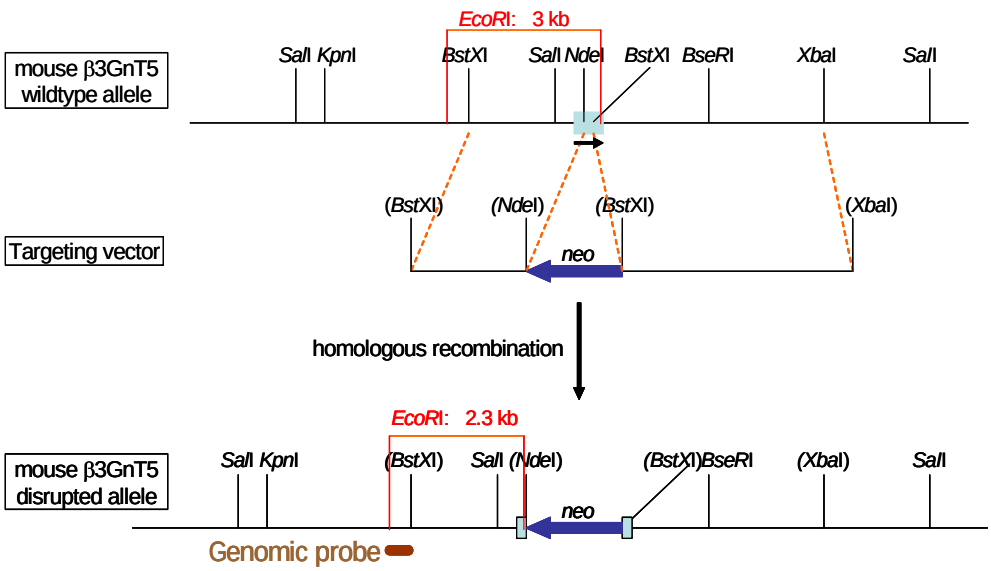


Figure 3 Biellmann *et. al.*

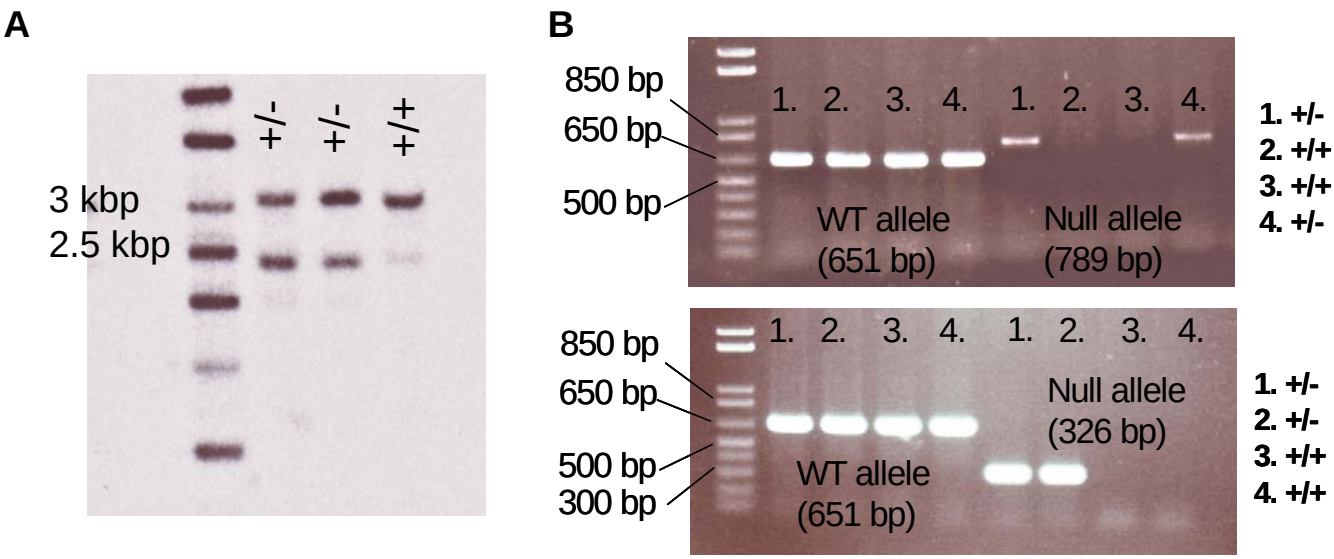


Figure 4 Biellmann *et. al.*

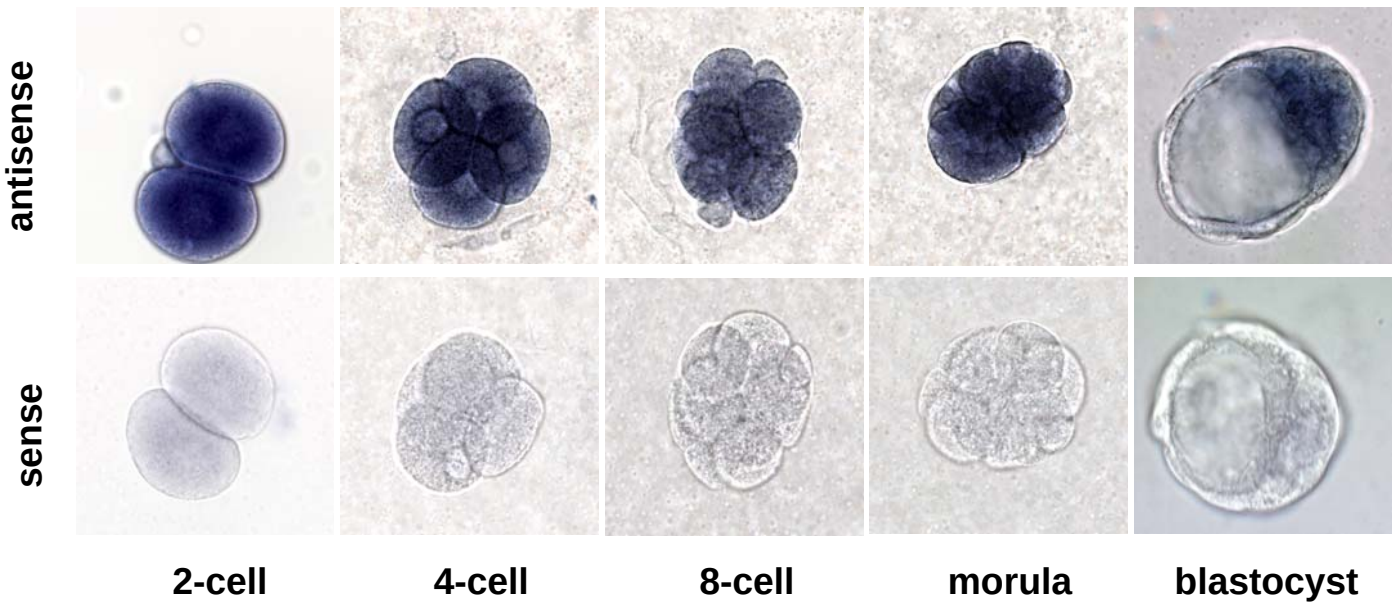
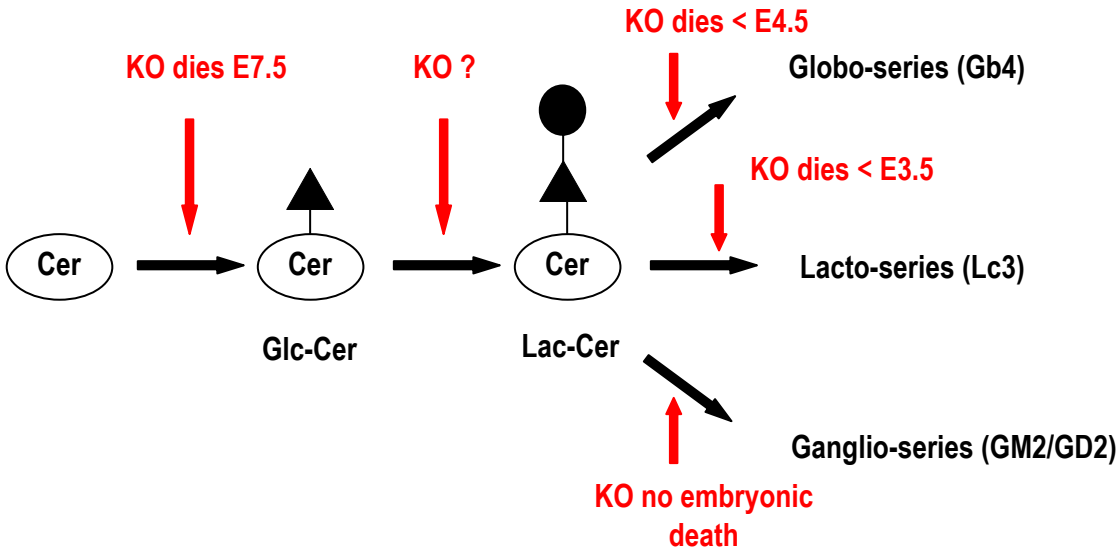
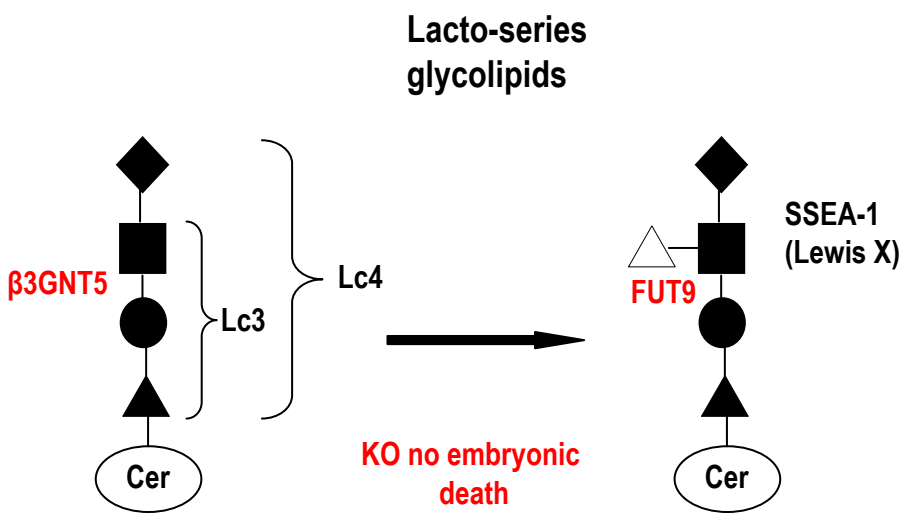


Figure 5 Biellmann *et. al.*

A.



B.



Supplementary Data:

The recipes for the buffers mentioned in the *in situ* protocol are as follows:

Hybridization Solution: store aliquots at -20°C

50% formamide

5x SSC

0.1% Tween-20

0.1% SDS

50 ug/ml E.coli tRNA

60 mM citric aid

In DEPC water

Filter 0.22 μM

Wash Buffers: store aliquots at -20°C

Solution I

50% formamide

5x SSC

37 mM citric acid

38 1% SDS

39 In DEPC water and filter as above

Solution II

50% formamide

2x SSC

93 mM citric acid

94 0.2% SDS

95 0.1% Tween-20

96 In DEPC water and filter as above

MAB Buffer (100ml): store aliquots at 4°C for 2-3 weeks

1.16 g maleic acid

1.74 g NaCl

0.7 g NaOH

0.1 ml Tween-20

Bring pH to 7.5 with NaOH and filter

Alkaline Phosphatase Buffer: **FRESH**

100 mM Tris-Cl pH 9.5

100 mM 5M NaCl

50 mM MgCl2

0.1% Tween-20

1% 100x levamisole

In DEPC water and filter

1. Discussion

Glycans impart greater complexity to the molecules they decorate than any other known modification. In essence, glycans diversify an already diverse set of proteins, lipids and other biological molecules. However, as with other biological systems, a certain amount of redundancy occurs. For glycosylation this is evident in each family of genes which catalyze the same linkages between the same donor and acceptor substrate units, as well as recycling of specific monosaccharide combinations with one another or other acceptor substrates. The connection between the proteome and the genome offered the first central dogma in Biology and is one example of how the proper function of two biological systems is inter- related and crucial to life. As more information and technologies become available to study glycans, glycomics will mature and offer an additional biological system to consider (fig.12) when profiling cellular and molecular interactions *in vivo*. The connection among glycome, proteome, and genome can offer us a more integrative understanding of biological complexity.

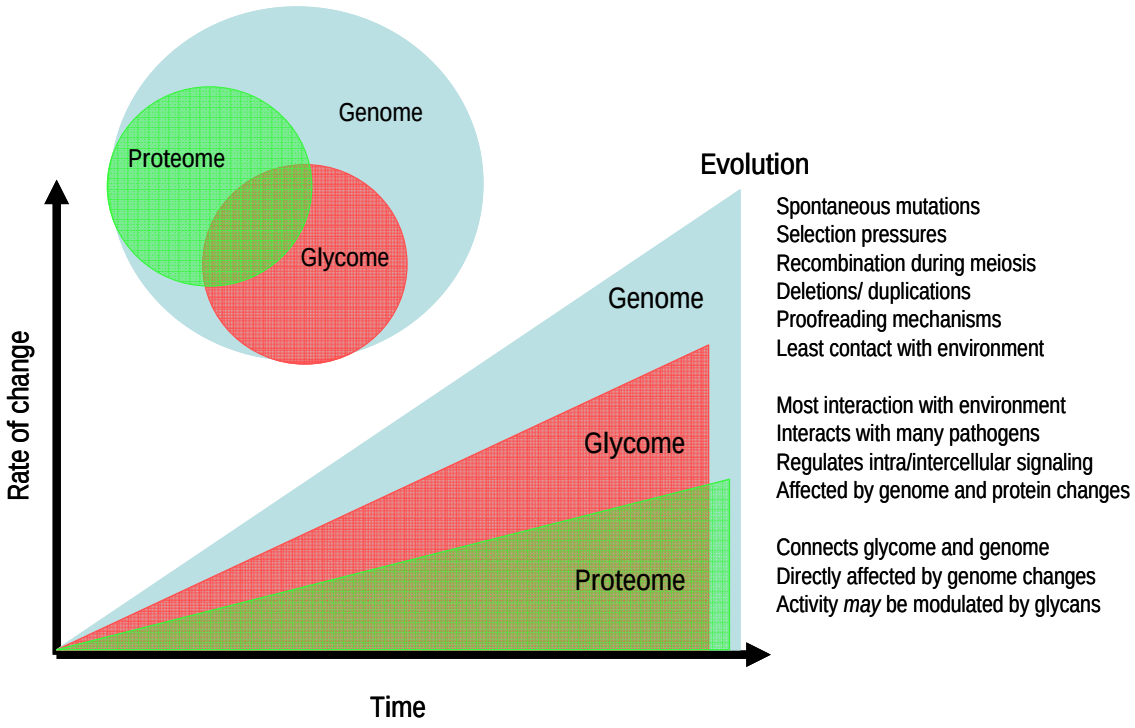


Figure 12: The interaction of genome, proteome, and glycome drives evolution.
The layered depiction of the relationship among the genome, glycome, and proteome represents their dependence upon one another over time. At the same time, the rates at which these three systems evolve over time necessarily differ due to their interactions. None of them actually ever stop 'evolving', but the proteome will always change in response to the genome which in turn responds to environmental pressures (perhaps due to glycome interactions) and/or spontaneous mutations. The glycome has the most contact with the environment in its interactions with pathogens. These systems have to work in sync to provide the best adaptation of an individual to its environment. In the long term this drives evolution.

To date, the ABO histo-blood group antigens (HBGAs) are probably the most studied glycan epitopes and can be used as an example to illustrate the interaction of the genome, proteome, and glycome. The ABO gene locus enables the production of the ABO antigens. There are three main allelic forms of the ABO gene locus. The A and B alleles control the expression of a GT, which catalyzes the addition of the final sugar residues specific to the A and B antigens. The A/B polymorphism is the result of four single nucleotide differences coded for in the ABO gene loci. These differences lead to the A and/or B specific GTs which only differ from each other by a few amino acids. In the case of the A antigen, the GT is an α1,3-GalNAc-transferase while for the B antigen it is an α1,3-Gal-transferase. In the rare event that alleles for both GTs are present, an AB phenotype is possible. The O antigen results when the GT is inactive and thus leaves the precursor H antigen unmodified. ABO antigens are found attached to oligosaccharides on glycoproteins and glycolipids that protrude to the surface of the red blood cells (RBCs) (fig.13). Finally, the HBGAs include other antigens coded for by the Lewis and secretor gene families in addition to the polymorphic ABO loci. (fig. 14).

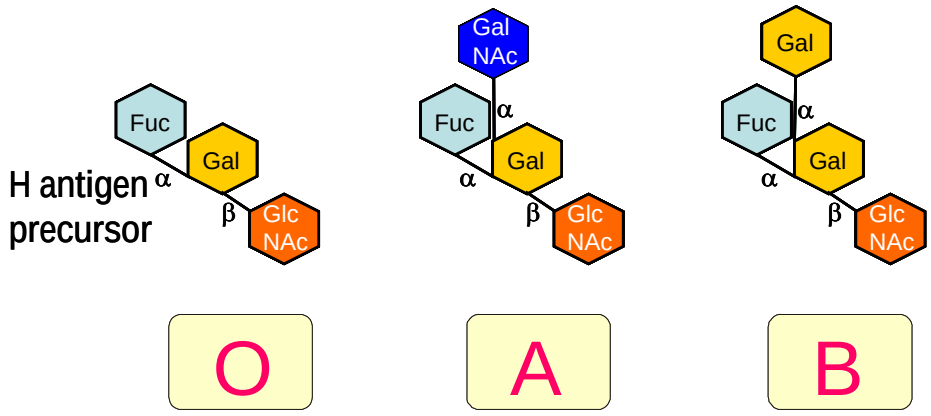


Figure 13: The ABO blood group structures.
The different structures of the ABO blood groups give them their serological specificities making them specific ligands for invading pathogens. The O antigen results from the unmodified precursor, H antigen. For A and B a GT resulting from the ABO gene polymorphic locus catalyzes the addition of the GalNAc and Gal, respectively, giving rise to the A and B antigens.

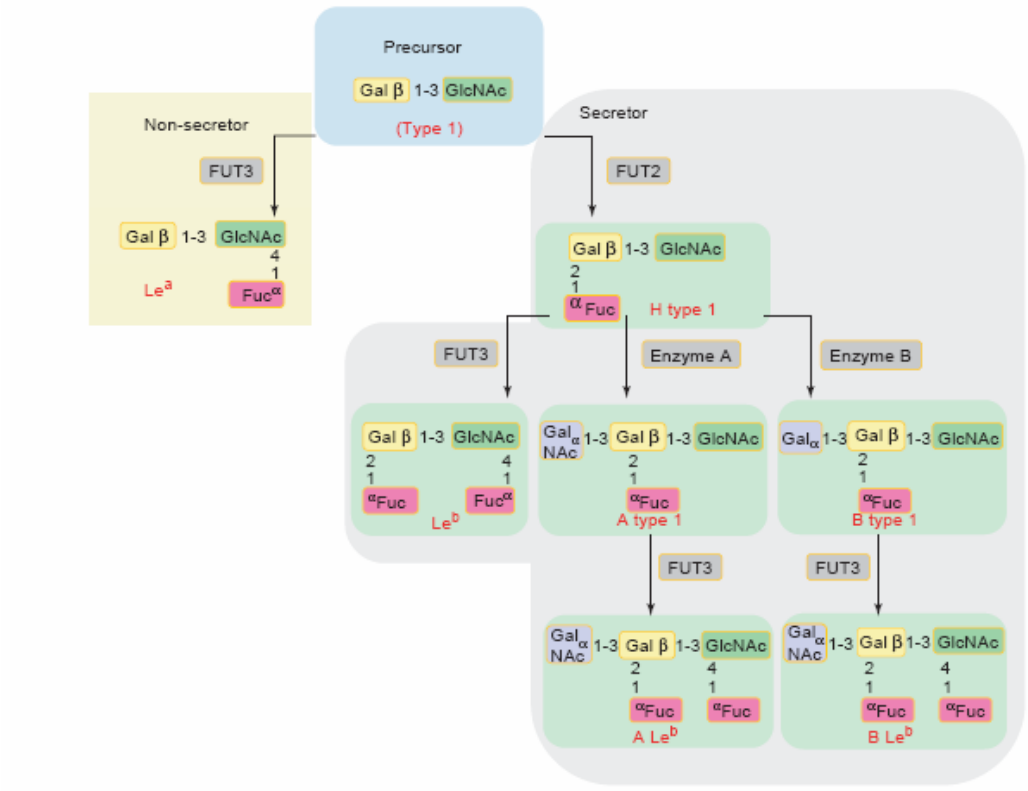


Figure 14: Glycan structure of the human HBGAs. The HBGAs are made up of the Lewis, secretor and ABO gene families. GTs control the synthesis of these antigens. Different expression of the GTs from the ABO polymorphic gene locus are predetermined, and specific to the individual. Since three gene families modulate the expression of the antigens, it is a highly diverse system with numerous combinatorial possibilities. (Taken from [1].

In an elegant mathematical model evolution of the ABO polymorphism is accounted for via intracellular and extracellular pathogen exposure [2]. In their model *Seymor et al. (2004)* show how the ABO polymorphism could have evolved, and account for the distribution of the four different phenotypes (A, B, O, AB) in the human population. The non-functional O allele is particularly striking as it is a selection for a 'loss of function' by not coding for a GT. While A and B antigen hosts readily produce antibodies against B and A antigens, respectively, both produce no natural antibodies towards the O antigen. The effects of intracellular and extracellular pathogens drive the ABO gene locus to different selective pressures. In the *Seymor et al. (2004)* model, for example, bacteria will adapt to rare host phenotypes or host alleles. This is a consequence of bacteria adapting more quickly to common host phenotypes and thus creating a frequency-dependent selection for rarer phenotypes and alleles. This helps maintain host polymorphism since several mutational changes are needed for the bacteria to adapt to particular host genotypes. Selective infections associated with bacteria can occur when these bacteria or their toxins use host glycans like the ABO antigens as entry sites for infection. Evidence for this

hypothesized glycan interaction comes from susceptibility studies in which a higher or lower rate of infection was correlated to the expression of the A, B, or O blood antigens. Although the molecular mechanisms behind these phenomena are not described, this list includes some of the historically most deadly bacteria such as *Vibrio cholerae* [3, 4] and *Shigella* [5].

For intracellular pathogens like viruses, it has been suggested that they can carry ABO antigens as part of their envelopes after infecting a host [6-9]. Hence, a virus released from an A host, carries A antigens, and will be recognized by the natural immune systems of O and B but not by A and AB hosts. However, viruses released from an O host are virtually unrecognizable by any immune system since O hosts lack terminal glycosylation. This means that a virus from an O host is more easily transmitted to A, B, or AB hosts than vice versa. As a result, host A, B, or O antigen expression can lead to different transmission rates among a host species depending on the specific antigen expressed by the virus and the host. A specific example of how a virus uses the ABO antigens as entry ports follows below. The point is, the ABO blood group system as we know it today protects subsets of individual of the population from transmitting infections caused by enveloped viral pathogens [8]. This interaction between pathogen and host carries consequences for both, as they are engaging in a race to survive.

The Norwalk virus (NV) has made use of histo-blood antigens as ligands for its target host cells and its molecular mechanism described. As mentioned the HBGAs include the ABO gene locus, the Lewis, and secretor gene families (for review see [10-12]). All of these are glycan structures (fig. 15). As a result certain individuals are at risk of being infected by certain strains of NV while others are not. The implication of the genetic factor in the infection rate of this virus was the focus of a study in the 1970's [1, 13]. For the first time it was shown that genetic factors in addition to acquired immunity was what predisposed a host for infection. Figure 15 illustrates how the NV is believed to interact with the HBGAs allowing for its specificity. The eight known binding patterns can be divided into two groups; the A/B and the Lewis (non-secretor) groups. This means all NV strains in the A/B binding group bind type A and/or B and O-saliva of the secretors, but not to the saliva of non-secretors. All NV strains in the Lewis group bind HBGAs of the non-secretors and type O-secretors with weak to no binding to type A or B secretors. Since the two groups mutually exclude A/B or the Lewis epitopes it is likely

that they represent two genetic lineages that evolved together with their human host although evidence for this is still inconclusive.

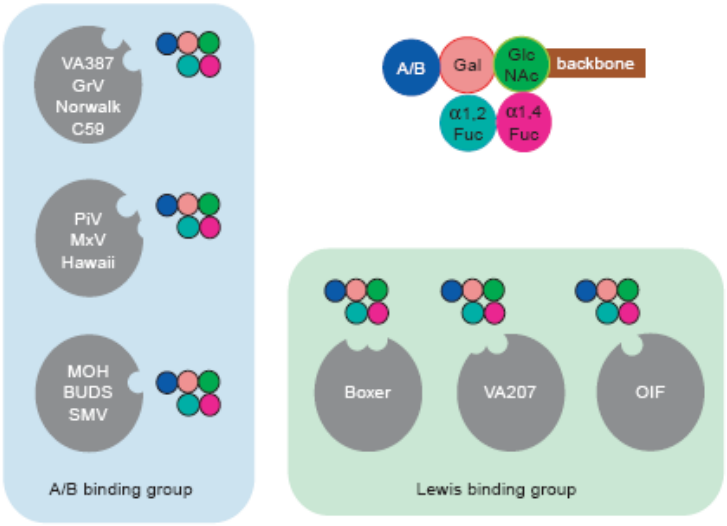


Figure 15: Model for six of the eight NV interactions with the HBGAs based on the Lewis and A/B groups. The structure on the top right shows a pentasaccharide final structure (ALe^b or BLe^b) of human HBGAs. Thirteen NV strains are indicated based on their relative interactions with the A/B H and Lewis epitopes. (Taken from [1])

For all their catalytic specificity, the GTs that control glycosylation are part of a biological system that drives diversity. The delicate balance necessary to provide an organism with the glycosylation it needs to survive and reproduce, while protecting it from a changing pathogenic environment is an enormous challenge. If we consider all of the functions glycans have within a complex multicellular organism and with other organisms the individual may come into contact with, the fine tuned nature of glycosylation becomes evident. Research has shown that glycans are extremely diverse, but also that the expression of certain glycans can be extremely specific and developmentally limited [14, 15]. The fetal and adult i and I antigens, respectively, discussed in the introduction are just one example of developmentally regulated glycan expression in mammals. How and why certain glycosylation patterns are so highly conserved if they are potential binding sites for pathogens is debatable and discussed briefly below. However, keeping as many variations as possible open for selection is advantageous for effective and 'multidirectional' evolutionary adaptation for the entire species.

Generally we distinguish between the glycans expressed on the cell surface and those within the cell. Even though glycans are common ligands for pathogen entry into the cell,

individual cell types have come to rely on specific glycans for their endogenous functions. Glycans within the cell often direct cell-type specific processes essential to cell survival. Disruption of these glycans can result in cell-specific defects. In humans a specific subset of genetic diseases called Congenital Defects of Glycoslyation (CDG) can result when the glycosylation machinery is somehow disrupted [16-21]. The effects of disrupting GTs involved in normal glycosylation pathways varies significantly among the various types of CDG described thus far. Patients may suffer from very mild to lethal phenotypes [22-25]. In addition other defects such as a loss of certain T-cell subsets and blood coagulation problems in mice missing a specific Sia-transferase [26]. This makes it difficult for certain glycan moieties to be discarded simply as a response to pathogen binding.

Since each cell type is dependent on the expression of a regulated and cell-type specific set of genes encoding GTs, these by definition must be evolutionarily favorable. Variations within a species exist, such as the ABO antigens in humans and primates, but tend to be limited. The glycan modification of conserved glycoproteins among related species may vary considerably [27]. Interspecies variations are generally more striking than intraspecies variation. Moreover, glycans can be eradicated from a species' repertoire completely. This was the case with terminal α-Gal epitopes in old world monkeys, apes and humans [28, 29] and the loss of N-glycolylneuraminic acid in humans [30]. This kind of dramatic loss precludes a superfluous or very well adapted alternative glycan taking over the function of the eliminated glycans.

Pathogens' ability to exploit or evolve to mimic or 'abuse' the glycans available in the host seems infinitely more likely than a complex multicellular organism constantly changing its glycome profile. However, Varki suggests that this is exactly what is happening. In his 'Red Queen effect' [8, 31] theory- a reference to the Red Queen in L. Carroll's *Through the Looking Glass*- Varki argues that the glycosylation within a particular individual is constantly being tampered with by parasitic, bacterial, and viral invaders. In order to keep up, these organisms must constantly evolve their glycome. The Red Queen tells Alice "it will take all the running you can do, to keep in the same place". Varki suggests that the glycome must constantly be changing and/or evolving effectively because it is in fact so dynamic. This necessarily affects evolution of the species (fig. 16).

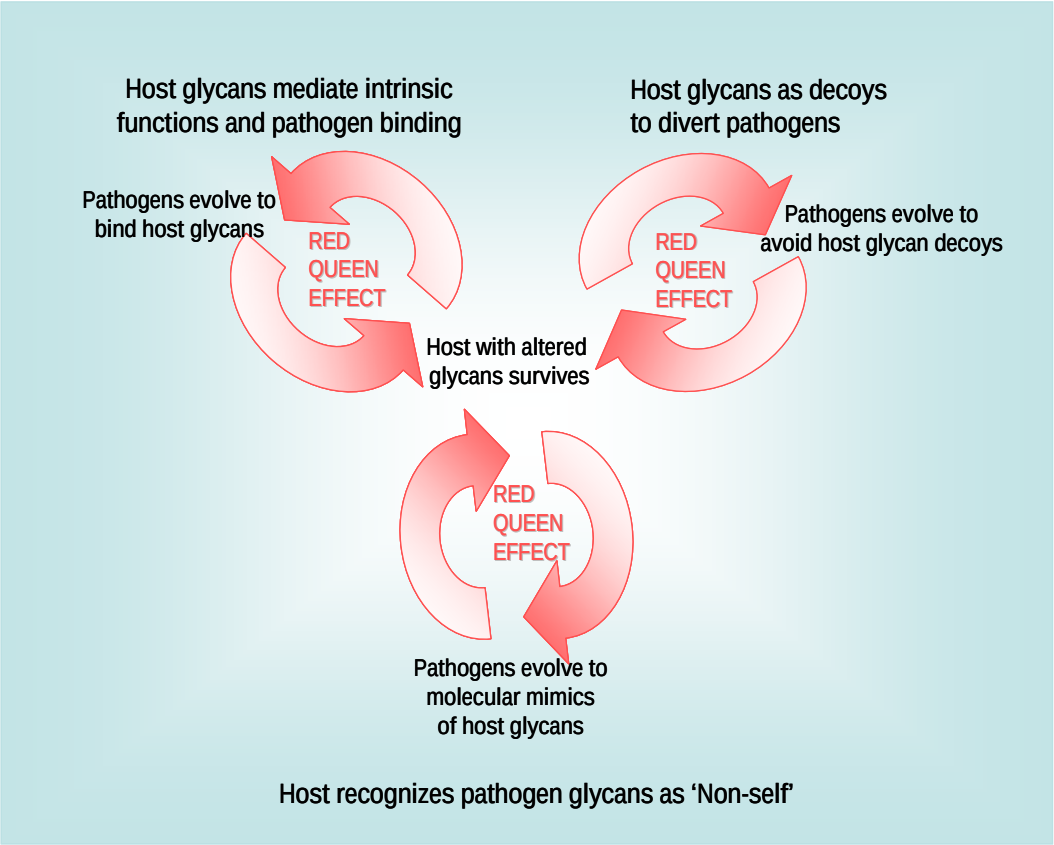


Figure 16: The Red Queen Effect according to Varki.(adapted from [31]) The circling arrows represent a potentially endless cycle between rapidly evolving potential pathogens and their target hosts. Although the host relies on glycans for proper endogenous functions, the host has to change them to evade pathogens glycan binding properties. Ideally these glycan 'adaptations' do not affect host cells function. Additionally hosts can synthesize decoys to divert pathogens away from cell surfaces. Normally, pathogen-specific glycans are recognized as 'nonself'. However, pathogens alter their surface glycans in an effort to mimic those found in the host ('self'). There are also possible Red Queen effects which involve host glycan binding proteins that recognize 'self'. In each cycle, hosts that alter their glycan repertoires without loss of cellular function are those which will survive.

2. Concluding remarks to GTs and the *B3gnt1* and *B3gnt5* null mice

Each member of the *B3gnt* gene family is described as having a preferred acceptor substrate. The phenotypic effects we expect when disrupting a specific GT are largely based on what we know about the expression of these acceptor substrates. We have observed the effects of disrupting *B3gnt1*and *B3gnt5 in vivo* in the murine model. Our results suggest that broader substrate specificity does not mean a more pronounced phenotypic effect should be expected. On the other hand, narrow substrate specificity does not imply a minimal phenotypic effect. This paradox underlines the importance of GTs and their products *in vivo*. These findings corroborate the idea that it is very difficult to predict the effects of altering glycosylation [32]. More importantly, GT activity can be developmentally indispensable [26, 33, 34] as in the case of *B3gnt5*. Table 2 below summarizes the phenotypes described for null mutant mice for various members of the *B3gnt* family.

Table 2: Phenotypes resulting from disrupted *B3gnt* genes

Gene (Protein) Name	Acceptor Glycan	Substrates	Expression Profile	Null murine phenotype
ignt (iGNT)	Gal	N-/O-Glycans	Fetal brain, all adult tissues	Not available
***B3gnt1/2* (β3GNT1)**	**Gal**	**N-/O-Glycans, Glycolipids, GAG**	**All adult tissues**	**1. Axon pathfinding in olfactory neurons disturbed [35] 2. Affects migration rate of GnRH-neurons [36] 3. Impaired male sexual behavior (Manuscript 1)**
B3gnt3 (β3GNT3)	Gal	O-Glycans Core 1 elongation	Colon, placenta, stomach, jejunum, and HEVs	1. higher in vivo rolling velocities of B and T lymphocytes 2. Decreased B-cells [37]
B3gnt4 (β3GNT4)	Gal	No Data	Brain	Not available
***B3gnt5* (β3GNT5)**	**Gal**	**Lactosylceramide Glycolipids**	**Placenta, testes, spleen, embryo**	**Causes pre-implantation embryonic death (Manuscript 2)**
B3gnt6 (β3GNT6)	GalNAc	Core-3 O-Glycans	Mucins in stomach, colon, and small intestine	Important for the modification of O-glycans which modulate susceptibility to colorectal tumors and colitis [38]

What makes it so difficult to predict phenotypes for GT disrupted mice? Like other enzymes which control the synthesis of protein and lipid modifying moieties, GTs mediate the production of complex structures throughout the entire organism. The effect of disrupting the activity of a single GT is two-fold since it is responsible for the modification of additional molecules. First, the loss of the modifying glycan can affect the interactive capabilities of the acceptor substrate. The avidity of the glycan hence plays a role in the phenotype dependent on GT activity. Just like antibody ligand epitopes, carbohydrates may be visible to their interactive ligand via very specific or more general features. As a result, only the specific disruption of this interacting structure will lead to a disturbance in the signaling cascade for the acceptor substrate. At the same time, the recognition structure may still be functional if it is independent of the sugar moiety under the control of the dysfunctional GT. Secondly; the acceptor substrate may or may not be modified by an alternative GT. Since there are multiple genes encoding enzymes with the same catalytic activity with the same donor and acceptor substrates, it is possible that some compensation can occur from a GT from the same family (fig. 17). However, the competitive activity of a GT with alternative donor specificity cannot be ruled out in the event of a gene disruption. Moreover, the data on acceptor substrate specificity for each GT may not be complete. GTs may have activities beyond those recognized from biochemical *in vitro* studies. Consequently the phenotypes we observe are often very different from those we expect based on previous data concerning the GT's acceptor substrates.

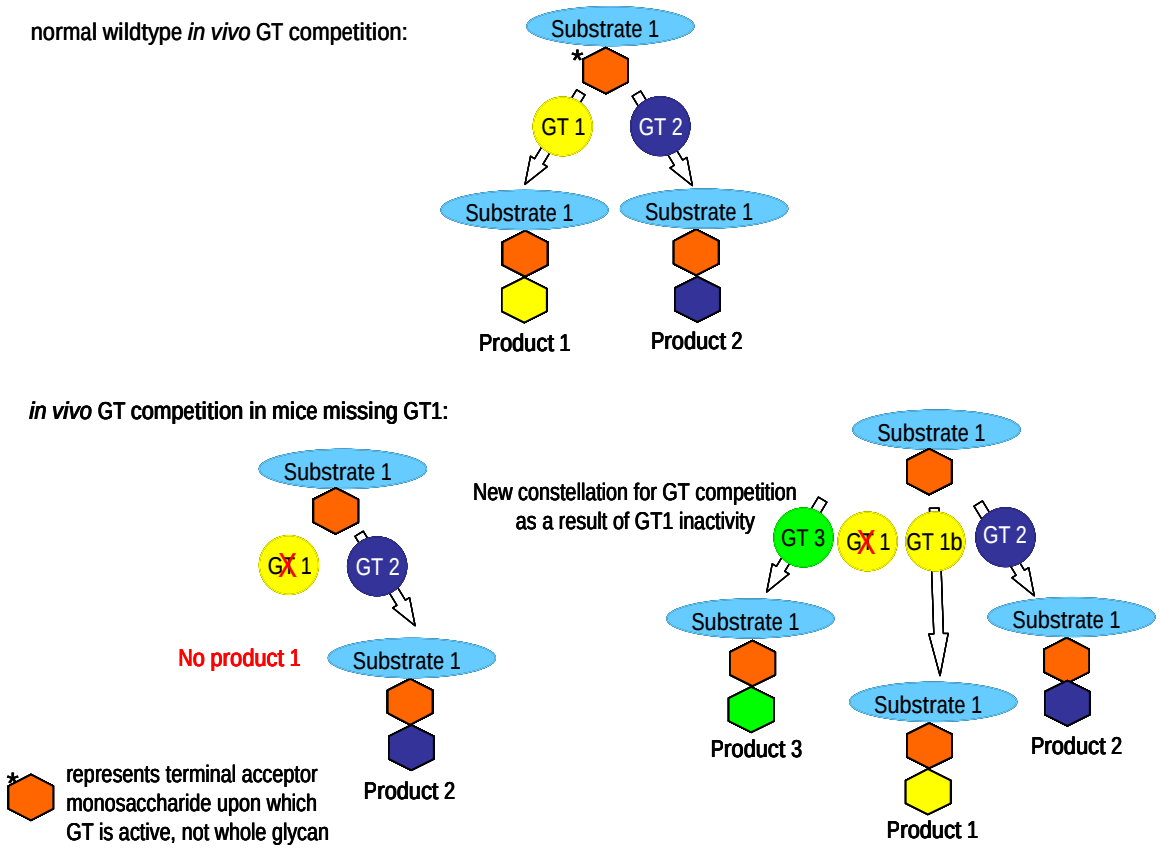


Figure 17: Acceptor substrate competition among GTs *in vivo*. Under wildtype conditions, the competing GTs are defined. There are several scenarios that can occur *in vivo* when a single GT is disrupted. Either there is a loss of the specific glycan moiety under the control of the disrupted GT, or another GT becomes active in its place. These new competing enzymes can either have the same catalytic activity (GT1b) or another one (GT3). As a result the effect of the disrupted GT (GT1) can range from no effect to very severe. The production of a new glycan structure due to new competing enzymes can have serious consequences for the acceptor substrates and their interacting partners.

All of these factors have to be considered when analyzing a GT null mouse. In *B3gnt1* null mice, we were able to show the loss of lactosamine chains in the OE, VNO, and T-cells in [35, 36]. However, this seemed to be developmentally limited to young mice. *Henion et al. (2006)* showed that lactosamines repopulated the olfactory bulb (OB) after postnatal day 15, suggesting a compensatory activity of another protein from the *B3gnt* gene family. Nevertheless, the initial complication in OB innervation and subsequent decreases in the repertoire of odorant receptors may contribute to the long term behavioral defects we observed in murine male sexual behavior. In addition to the abnormal sexual behavior of the males, another behavioral phenotype was observed in the *B3gnt1* null female mice. They were often seen eating viable new born offspring. Although this behavior was not specifically investigated, it was regularly observed. It is likely that the females are not able to recognize their own offspring due to an olfactory

deficit similar to the one detected in the null males. This might have compounded the problems we had breeding the null animals.

The pre-implantation embryonic lethal phenotype observed when *B3gnt5* was disrupted came quite unexpectedly. Mice missing the glucosylceramide transferase (*Ugcg*), a gene responsible for providing the precursor for Lc3 synthase, also caused embryos to die. However, the embryos were viable until E6.5-7.5 [39, 40]. However, while we show continuous expression of *B3gnt5* at all stages leading up to the blastocyst *Yamishita et al.* describe a lack of expression of *Ugcg* at the 2 and 4-cell stage although they claim it is present in unfertilized eggs. There are many ways to interpret these two apparently conflicting results if we assume maternal mRNA encoding β3GNT5 is not interfering, but some are more likely. First, there is an unknown homolog for *Ugcg* being expressed in the mice missing this gene. Secondly, β3GNT5 is acting on pre-existing glucosylceramide obtained from the parents. Thirdly, the possibility of additional activity of the β3GNT5 on another substrate cannot be excluded at this time. However, mice missing other glycolipid synthase genes have been described which are lethal before E3.5 [41]. In the case of Gb4 synthase the Glc-Cer structure is also necessary for the activity of the Gb4 synthase. There are many unanswered questions when it comes to the functions of glycolipids *in vivo*. Clearly we have only scratched the surface in this complex and relatively young field. Until we can describe all glycolipids and identify the genes which modify them it will be difficult to interpret the results we have obtained from murine models so far. Perhaps the lethal phenotype we observed in the *B3gnt5* null mice is not linked to the synthesis of glycolipids at all. Further investigations of the catalytic targets and expression of *B3gnt5* are necessary to examine these hypotheses.

Mice with inactivated GTs have shown that GTs are not just interchangeable isozymes [35, 42-50]. However, the fine line that gives each GT its specific role *in vivo* is still poorly understood. To study the GTs effectively, closer examination of the mechanisms controlling their expression and their acceptor substrates will be necessary before null mice can be conclusively fully phenotyped. Both *in vitro* biochemical studies and breeding of viable GT null mice with other null models for suspected interactive partners could provide insights into specific GT activity. Furthermore, breeding mice which are null for two different members of the same GT family might also give us a better idea of which enzymes can and will compensate if one is rendered dysfunctional. GTs mediate the

production of a highly diverse set of biological structures. Unraveling this complexity requires continued systematic *in vitro* and *in vivo* investigations.

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Curriculum Vitae

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University and College Education

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2002- date:	Biological Sciences PhD Student (Dr. sc. nat.) **Institute for Physiology, University of Zurich** Prof. Dr. T. Hennet
9.2000	**Masters Thesis (Grade: 1.5 very good)** Institute for Transplantation Immunology University of Heidelberg, Germany (Prof. Dr. med P. Terness) „Development of an Affinity Chromatography Column for the One-Step Purification of *gp96* with Single-Chain Antibodies"
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Publications

1. Henion, T.R., Raitcheva, D., Grosholz, R., **Biellmann, F.**, Skarnes, W.C., Hennet, T., Schwarting, G.A. (2005). "β1,3-N-Acetylglucosaminyltransferase 1 Glycosylation Is Required for Axon Pathfinding by Olfactory Sensory Neurons *J. Neurosci.* **25**(8): 1894-1903.

2. **Biellmann, F**., Henion, T., Bürki, K., Hennet, T. (accepted July 2007) "Impaired sexual behavior in male mice deficient for the "β1,3-N-Acetylglucosaminyltransferase –1 gene" *Mol. Reprod. Dev.* (In press)